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TRYPTOPHAN AS A BACTERIOPHAGE ADSORPTION FACTOR

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THE bacteriophage which is the subject of this study was isolated from sewage by serial subculture on the *Salmonella typhi* strain F1 growing in nutrient broth. This strain is not attacked by many of the phages active on typhoid and only slightly by " anti-O " phages 1 and 2. The new phage was found to have a very wide host range among strains of *S. typhi*, but no action on four strains of *Bact. coli* including the American Coli B. When propagated on a simple ammonia medium, it was found to require tryptophan for adsorption, as was also the case with the T4 phage acting on Coli B (Anderson, 1948 *a*, *b*, *c*).

Anderson also found that this phage could be activated, though less efficiently, by phenylalanine, diiodotyrosine and tyrosine, while other synthetic amino acids having aromatic groups were also active. "Oddly enough", norleucine was slightly active.

In the present case attention has been directed more to the natural amino acids in DL-form and it has been found that phenylalanine is about 30 times less active than tryptophan ; tyrosine about 80 times, and leucine, isoleucine, norleucine, norvaline, methionine and S-methyl cysteine about 1000 times. Cystine, cysteine, valine and all others were inactive at 1000 times greater concentration than trytophan.

The present work, however, is confined to tryptophan operating in media which, at any rate initially, were free from other amino acids.

A demonstrable effect of tryptophan was obtained with 0·1 μg.per ml. L-tryptophan and a maximal effect with 10 μg. per ml. These figures are of the same order as found by Anderson with his T4 phage on Coli B.

The new phage soon acquired the power to dispense with tryptophan if added to a culture growing without added tryptophan. It was supposed that the tryptophan requiring phage ($tryp^+$) contained genetic mutants which did not require tryptophan for adsorption ($tryp^-$) and that these were selected on propagation without added tryptophan.

Two main tests were applied for distinguishing a $tryp^+$ phage from a $tryp^-$ mutant. A $tryp^+$ phage should show no measurable adsorption when purified

and suspended in buffer with washed cells, but should show rapid adsorption on addition of tryptophan. A tryp$^-$ phage under the same conditions should adsorb at the same rate in the presence or absence of tryptophan. Further a tryp$^+$ phage should produce plaques in high dilution on plates of a minimal medium containing added tryptophan, but no plaques on tryptophan-free plates, except in so far as it contained tryp$^-$ mutants. From the counts on the two plates, the phage could be described as having a ratio tryp$^+$: tryp$^-$ of (say) $1 \times 10^4 : 1$.

A tryp$^-$ phage should under these conditions give equal counts with or without tryptophan, in which case the co-existence of tryp$^+$ phages in small numbers could not be determined.

It will be seen that the behaviour of the tryp$^+$ component of the phage was in accordance with expectation, but the tryp$^-$ mutant appeared to be an ideal which could not be attained. All preparations adsorbed more rapidly on adding tryptophan and tryp$^+$: tryp$^-$ ratios varied from $> 1 : 1$ to $< 1 : 1$, the lowest being $1 : 3{\cdot}5 \times 10^3$. The tryp$^-$ preparations thus appeared to be less homogeneous than the tryp$^+$.

METHODS

The bacterium used was chiefly *Salm-typhi* " Bact. 1 " (Fildes, 1956).

Minimal culture medium used for differentiation of the two varieties of phage

The details of this were given by Kay and Fildes (1950). In the present work the glucose was reduced to 0·01 M and L-cystine was added to 0·0005 M.

For plate cultures 1·5 per cent agar was added and 0·01 M citrate. DL-tryptophan was included as required at 0·0001 M.

Plating methods

Surface drops following the principle of Fildes and Smart (1926) were used exclusively. Plates were dried on a warm plate before and after flooding on the lawn of Bact. 1. The suspension used was made in buffer from a deposited culture in minimal medium. The drops of phage suspension were also dried on the plate. Plaques could be counted to 100–120 in 1 drop (0·025 ml.) on overnight reading by means of a × 10 binocular microscope and transmitted oblique illumination.

Adsorption procedure

In general the method of Fildes and Kay (1955) was followed. Bacteria were grown in minimal medium, washed 4 times and suspended in buffer to give a final reading 0·150 on Hilger's Spekker Absorptiometer. All diluents contained human albumen. Phage in the mixtures was 5×10^5 per ml. Samples were diluted 1/100 and supernatants plated.

The question of undisclosed tryptophan in cultures

If the bacteria used for a culture are washed it may be supposed that initially the " minimal " cultures are free from tryptophan but Fildes (1956) has shown that the bacteria used in the present work produce in the course of growth sufficient tryptophan to permit the syntrophic growth of a tryptophan-requiring coli mutant. This being so, it may be supposed that sufficient tryptophan is produced to allow adsorption of a tryp$^+$ phage in a liquid culture. A further technical difficulty arises from the effect of lysis. Since Fildes (1956) also showed that tryptophan could be extracted from washed cells, it is highly probable that it is liberated on lysis by phage, and since a gradually increasing lysis results from the propagation of tryp$^-$ mutants in the absence of added tryptophan, a gradually increasing concentration of tryptophan may also affect the propagation of the tryp$^+$ phages.

On the surface of agar containing no amino acids a tryp$^+$ phage does not produce a plaque. From this it is concluded that tryptophan is not released by the lawn in sufficient concentration at an early enough time to allow adsorption and a series of bursts to produce

a visible plaque before the area is overgrown. Nevertheless it is likely that propagation of tryp$^+$ phage does in fact take place in the absence of added tryptophan, though the results are invisible.

For these reasons experiments with growing cultures are always subject to complication.

Determination of ratios tryp$^+$: tryp$^-$

Tryp$^+$ lysates.—These preparations consisted of purified suspensions of phage in buffer-albumen containing a crystal of thymol. The procedure was to make serial 10-fold dilutions in buffer-albumen and drop 3 to 6 drops of each dilution on minimal plates with and without 0·0001 M DL-tryptophan and a lawn of Bact. 1. Next day the number of plaques per ml. was counted on the tryptophan-containing and -free plates. With a tryp$^+$ phage only a small number appeared in the low dilutions on the tryptophan-free plates. These were accepted as an estimate of the number of tryp$^-$ phages. The tryp$^+$ phages were given by the count on the tryptophan plates *minus* the tryp$^-$. A ratio $+ : -$ was then calculated.

Tryp$^-$ lysates.—In these cases the counts on the tryptophan and tryptophan-free plates varied only slightly. Since the counts on tryptophan-free plates had on rare occasions been as much as 20 per cent higher than on the parallel tryptophan-containing plates, differences of this order were taken to have no significance. It was clearly impossible by these means to determine the proportion of tryp$^+$ phages in a lysate if tryp$^-$ phages were in similar or greater concentration. At most it could be said that the tryp$^+$: tryp$^-$ ratio was less than 1 : 1.

When the ratio tryp$^+$: tryp$^-$ was found to be $< 1 : 1$, the following method was used to determine the proportion of tryp$^+$ phages in a lysate :—

The general procedure of the adsorption experiment was followed. The phase was diluted so that a calculated number per ml. was present in each of 2 tubes. This was usually 100 times more than in the normal adsorption experiment. One tube contained bacterial suspension about 50 per cent more concentrated than usual. The tubes were allowed to adsorb for 1 or 2 hr., after which time the tube containing suspension was centrifuged and the unadsorbed residue in the supernatant tested. The input of phage was estimated at the end of the experiment by plating in dilutions 1 in 10 to 1 in 10^4 with and without tryptophan. The supernatant of the residue was plated in the same way but also undiluted.

The counts of the input with and without tryptophan were so similar that differences were neglected and the count with tryptophan taken as the total input. The count of the residue *plus* tryptophan gave the fraction of the input which was not adsorbed. Thus the difference between these counts of input and residue gave the number of tryp$^-$ phages. The residue however contained phages which did in fact adsorb on a plate without tryptophan and these were taken to be tryp$^-$ phages which had failed to adsorb in 1–2 hr. The difference between the 2 counts of the residue thus gave the number of tryp$^+$ phages and the ratio of these to the tryp$^-$ input was then determined.

If the input was 1×10^7 and the residue 1×10^5 of which 2×10^4 were tryp$^-$, there must have been 8×10^4 tryp$^+$ phages in the original phage giving a $+ : -$ ratio $= 1 : 124$.

Plaques

Sampling of plaques can only be carried out profitably when the required variety is in marked excess. The plaque was sucked with a Pasteur pipette and dispersed into 3 ml. buffer-albumen. Some 10^7 phages were usually obtained. Four serial 10-fold dilutions were made and centrifuged. The supernatants were then plated with and without tryptophan. The object of the centrifuging was to remove adsorbed phages which would produce plaques whether tryptophan was present or not.

Plaque sizes

The plaques produced by both tryp$^+$ and tryp$^-$ phages in the presence of tryptophan on minimal plates were clear areas, measuring 0·5 to 0·3 mm. in diameter. There were however a varying number of smaller plaques, of the order of 0·15 mm. Tryp$^-$ plaques without tryptophan showed no obvious difference. On the other hand, the residue left after tryp$^-$ phages had been removed by adsorption without tryptophan, when plated without tryptophan, showed a larger number of small plaques, in fact some so small as to have no clear centre and recognizable with a $\times$ 10 binocular microscope only as a punctate object with a tinge of yellow which seemed to be characteristic of this phage. Since this

appearance was only seen in the absence of added tryptophan it was concluded that it was due to lack of sufficient tryptophan to produce a normal plaque, but sufficient tryptophan synthesized by the lawn to produce an abortive plaque.

These plaques have been looked upon as tryp$^-$ phages unadsorbed by chance in the course of the experiment, but on the other hand they may be partially tryp$^+$ capable of producing a visible result with the aid of a quantity of synthesized tryptophan which is inadequate for a fully tryp$^+$ particle.

Purification of phages

Phages were always purified to remove tryptophan or other adsorption co-factors by slow-high-slow-speed centrifuging, the high speed being repeated for two washings in buffer-albumen.

In low titre specimens from experiments, losses during these manipulations were much reduced by using coliphage T2 killed by heating at 70° for 30 min. as a carrier.

EXPERIMENTAL

Characters of a Tryp$^+$ Phage

A single plaque taken from a high dilution of a previous lysate, grown in minimal medium *plus* tryptophan, produced a lysate of Bact. 1, which when purified and concentrated contained 6×10^{10} phages per ml.

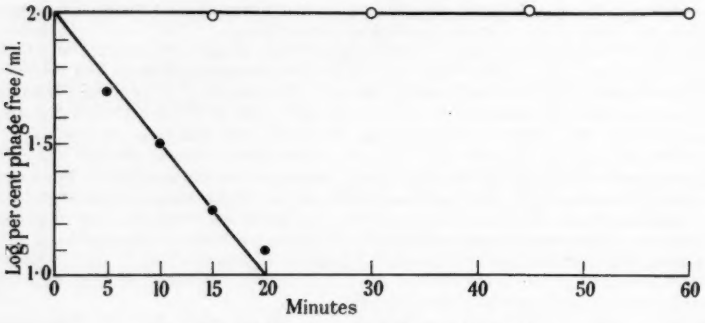

FIG. 1.—Adsorption rates of a tryp$^+$ phage.
● = with tryptophan, ○ = without.

Adsorption.—Fig. 1 shows that these phages were not adsorbed in 60 min. without tryptophan, but with tryptophan 87 per cent were adsorbed in 20 min. at approximately a constant rate.

Ratio +/−.—The + : − ratio of this lysate was determined: The phage at 1×10^{10} per ml. was dropped undiluted and diluted 1 in 10—10^8 on plates *plus* and *minus* tryptophan. There were 4 drops per dilution.

On tryptophan plates 102 plaques were counted in 4 drops of the 1 in 10^7 dilution, and with no tryptophan 130 plaques in 1 in 10^3 dilution.

Thus *plus* tryptophan there were in the lysate $102 \times \frac{40}{4} \times 10^7 = 1{\cdot}0 \times 10^{10}$ phages per ml. and without tryptophan $130 \times \frac{40}{4} \times 10^3 = 1{\cdot}3 \times 10^6$. The + : − ratio was therefore $7{\cdot}7 \times 10^3$.

Five separate plaques were taken from a high dilution of this lysate and the ratios determined. These were $2{\cdot}4 \times 10^5$, 4×10^4, $1{\cdot}3 \times 10^5$, $2{\cdot}5 \times 10^4$, $8 \times 10^4 : 1$.

Passage.—This lysate propagated with tryptophan maintained the same characters. Fig. 2 shows that under these conditions the free tryp$^+$ phages and the free tryp$^-$ mutants increased at approximately the same rate and so maintained the ratio. The irregularities were largely due to the necessity to purify each sample for test, which introduced varying losses.

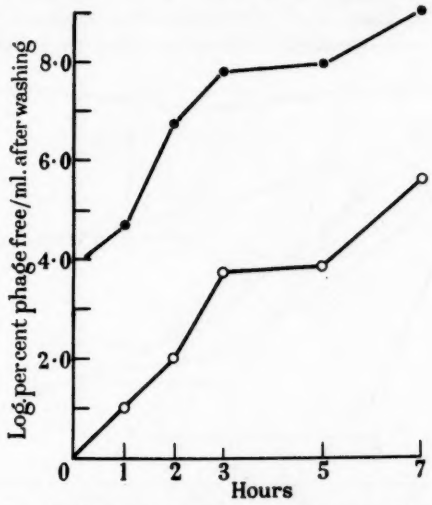

FIG. 2.—Production rates of a tryp$^+$ phage growing in tryptophan and of its tryp$^-$ mutant, as shown by plating with (●) and without (○) tryptophan.

Propagation of a Tryp$^+$ Phage Without Tryptophan

When a tryp$^+$ phage was propagated in series without tryptophan its character changed. Fig. 3 shows the result of 3 serial subcultures, each lysate being purified before continuing to the next. The original phage gave the typical adsorption of a tryp$^+$ phage. The first propagation without tryptophan showed 50 per cent adsorption without tryptophan in 17 min., the 2nd in 10 min. and the 3rd in 6 min.

The rates of adsorption without tryptophan were not constant, suggesting that the lysates were not homogeneous, although when tested with tryptophan the log per cent free approximated to a straight line. This lack of homogeneity was no doubt due to selection of tryp$^-$ mutants. The ratios tryp$^+$: tryp$^-$ fell as follows :

Original . .	$. 5 \times 10^4 : 1$
1st propagation . .	$2 : 1$
2nd ,, . .	$< 1 : 1$
3rd ,, . .	$< 1 : 1$

The rate of adsorption in the 3rd propagation did not suggest that the phage had already become fully tryp⁻ and the + : — ratio was calculated by the adsorption method. The phage was adsorbed for 1 hr. without tryptophan and left a residue $9{\cdot}3 \times 10^3$ which consisted of $6{\cdot}1 \times 10^2$ tryp⁻ phages and $8{\cdot}7 \times 10^3$ tryp⁺ i.e. a ratio + : — = 14 : 1. The preparation before adsorption contained $1{\cdot}4 \times 10^6$ phages, suggesting that it consisted of $1{\cdot}31 \times 10^6$ tryp⁻ phages and $8{\cdot}7 \times 10^3$ tryp⁺ giving a + : — ratio = 1 : 131.

The mechanism of the selection exerted by lack of added tryptophan is shown in Fig. 4. Here a tryp⁺ phage, with ratio 1×10^4 : 1, was added to 1×10^4 per ml. to a suspension of Bact. 1 in tryptophan-free medium. The bacteria used were deposited from an overnight culture to remove synthesized tryptophan.

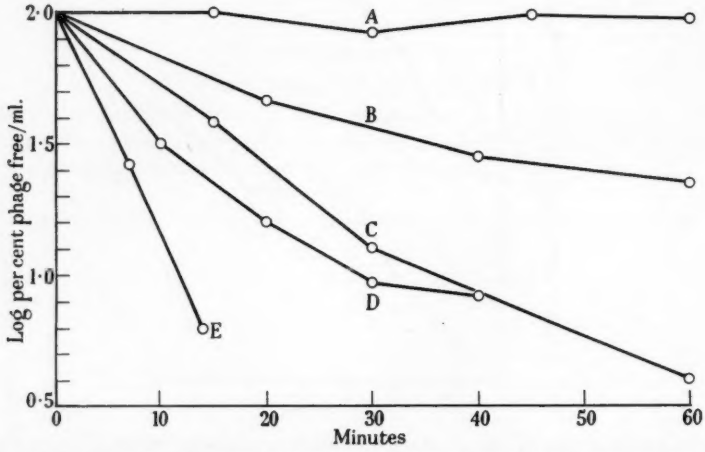

FIG. 3.—Adsorption rates of a tryp⁺ phage without tryptophan (A) and after one (B), 2 (C) and 3 (D) serial subcultures without tryptophan. E = rate of A with tryptophan and is equivalent to the rates of B, C, D with tryptophan.

In 5 hrs. the Spekker reading rose from 0·100 to 0·650 with no demonstrable lysis. Samples were removed at intervals and the supernatants purified. As already noted, this purification introduces irregularities into the counts. The supernatants were next plated with and without added tryptophan.

It will be seen in Fig. 4 that the tryp⁻ mutants grew from the start at a more or less constant rate, while the tryp⁺ phages fell up to 3 hr. and then rapidly increased. The behaviour of the tryp⁺ phages is ascribed to an initial failure to adsorb followed by adsorption resulting from tryptophan released by the growing bacteria and tryp⁻ bursts.

The 3 hr. interval of no production of tryp⁺ phages clearly allowed the tryp⁻ mutants to obtain an advantage, so that in 3 hr. the ratio + : — had fallen from 1×10^4 : 1 to 1 : 1, although subsequently it increased again to 6×10^2 : 1.

Isolation of Tryp⁻ Phages by Plaque Selection

Continued propagation of a tryp⁺ phage in the absence of added tryptophan did not produce a lysate which was not obviously mixed owing to the inevitable

growth of tryp$^+$ phages. On the other hand when, by this method, a ratio $+ : - < 1 : 1$ had been obtained, plaque selection of tryp$^-$ phages became practicable.

A " mixed " phage, with a ratio $< 1 : 1$ and a curved line adsorption without tryptophan was plated in high dilution without tryptophan and a single plaque taken to the same medium. The eventual lysate " A " gave a straight line adsorption with increased rate *plus* tryptophan.

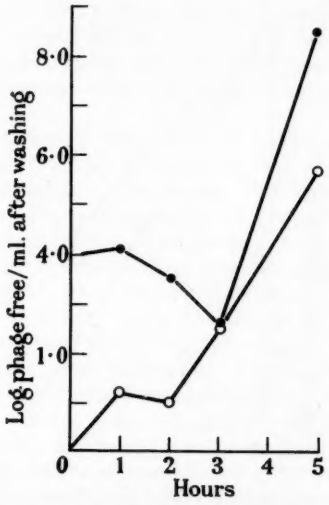

FIG. 4.—Production rates of a tryp$^+$ phage growing without tryptophan and of its tryp$^-$ mutant, as shown by plating with (●) and without (○) tryptophan.

This phage " A " was analysed by the adsorption method :

Phage plated *plus* tryptophan	$6{\cdot}2 \times 10^7$
,, ,, without ,, ...	$5{\cdot}0 \times 10^7$

The difference between these was not considered significant.

Residue after adsorption plated *plus* tryptophan . .	$1{\cdot}5 \times 10^5$
,, ,, ,, ,, without ,, .	$6{\cdot}7 \times 10^4$

Thus $6{\cdot}18 \times 10^7$ were taken to be adsorbable tryp$^-$ and $1{\cdot}5 \times 10^5$ unadsorbable of which $8{\cdot}3 \times 10^4$ were tryp$^+$, giving a $+ : -$ ratio 1 : 744.

This lysate " A " was plated in high dilution and plaques selected twice in series from which another lysate " B " was prepared without tryptophan.

The rate of adsorption of this preparation " B " is shown in Fig. 5. Without tryptophan the log per cent free at intervals approximated to a straight line, but the rate was still obviously increased by added tryptophan. The analysis of this phage gave :

Phage plated *plus* tryptophan	$5{\cdot}5 \times 10^7$
,, ,, without ,, ...	$3{\cdot}7 \times 10^7$

This difference was not taken to be significant.

Residue after adsorption plated *plus* tryptophan . .	$2{\cdot}1 \times 10^4$
,, ,, ,, ,, without ,, .	$5{\cdot}3 \times 10^3$

Thus $5{\cdot}49 \times 10^7$ were adsorbable tryp$^-$ and $2{\cdot}1 \times 10^4$ not adsorbable of which $1{\cdot}6 \times 10^4$ were tryp$^+$, giving a ratio $+ : - = 1 : 3{\cdot}5 \times 10^3$.

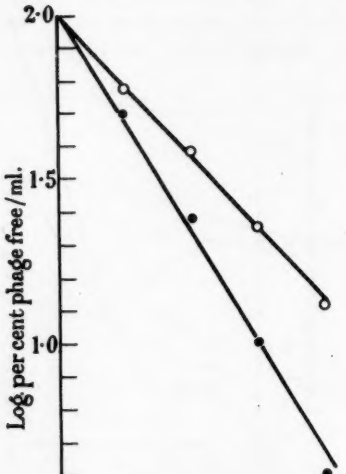

FIG. 5.—Adsorption rates of a tryp$^-$ phage with (●) and without (○) tryptophan.

A $+ : -$ ratio $1 : 3{\cdot}5 \times 10^3$ may be looked upon as a reasonable approximation to a tryp$^-$ phage, but even in this case one of the criteria of a tryp$^-$ phage was not achieved. The rate of adsorption of the phage was still accelerated by tryptophan, and the acceleration could not be due to an action on the small number of tryp$^+$ phages present. The action of tryptophan must also be exerted on those which have been described as tryp$^-$ units.

DISCUSSION

Reconsideration of the Status of Tryp$^-$ Phage

The conception of the difference between a tryp$^+$ phage and a tryp$^-$, as set out in the beginning of this paper, appears to require some modification.

The tryp$^+$ phage certainly propagates in the same form in added tryptophan and does not adsorb in buffer in the absence of added tryptophan. It does not produce visible plaques on a plate in spite of the inevitable production of tryptophan by the lawn, this no doubt being due to the production being too late or

too small to satisfy requirements. In high concentrations of the phage plaques appear on a plate without added tryptophan and these have been described as tryp$^-$ mutants numbering about one in 10^4 tryp$^+$. These mutants grow in a liquid culture with added tryptophan at the same rate as the tryp$^+$ and therefore the lysates tend to contain the same proportions of the two.

The behaviour of the tryp$^+$ phage is thus in accordance with expectation but complications arise when it is grown in the absence of added tryptophan. The chief complication is due to tryptophan released by the growing or lysing bacteria which allows, after an initial delay, the tryp$^+$ components to grow, thus reducing the selective pressure in favour of the tryp$^-$ mutants. However, by repeated propagation without added tryptophan, the tryp$^-$ components can exceed the number of tryp$^+$. At this stage plaque selection can be carried out and a tryp$^-$ phage propagated.

It is however found that this phage from a single plaque when adsorbed on Bact. 1 in the absence of tryptophan leaves behind an unadsorbed residue consisting of more tryp$^+$ than tryp$^-$ components. It can thus be calculated that a tryp$^-$ phage from a single plaque contains up to 1 tryp$^+$ component in $3{\cdot}5 \times 10^3$ tryp$^-$. When the rate of adsorption of these phages is tested with and without tryptophan, it is found that tryptophan accelerates more than could be ascribed to the small content of tryp$^+$ components. The tryp$^-$ phage is therefore not indifferent to tryptophan, but merely more indifferent than a tryp$^+$ phage. When the residue left after adsorption without tryptophan is plated in the absence of tryptophan it is noteworthy that a number of plaques, counted as tryp$^-$, are extremely small and punctate. They are quite different from the frankly tryp$^-$ plaques and are not far removed from " invisible " tryp$^+$ plaques. It seems that in these cases most of the frankly tryp$^-$ phages having been removed during the adsorption, the residue contains a number of phages which are incapable of producing a normal size plaque, but can just operate by means of the tryptophan produced by the lawn, which, however, is not in sufficient concentration to allow a frankly tryp$^+$ phage to produce a visible result. Thus it appears that from a frankly tryp$^+$ phage, which adsorbs only when the tryptophan concentration is above a certain figure, there is a series of components which adsorb poorly and others less poorly and finally quite well without added tryptophan but not to an optimal degree.

This conclusion of a graduated series of phages from tryp$^+$ to tryp$^-$ requiring less and less the addition of tryptophan is similar to that of Anderson who described a series showing an increasing efficiency in adsorption in the absence of tryptophan.

Hypothesis on Difference Between the Two Phages

Anderson has claimed that the variety here called a tryp$^+$ phage is " activated " by tryptophan above a certain concentration and deactivated again on mere dilution. A purified tryp$^+$ phage is, therefore, always inactive. Since tryptophan has also a measurable action on a tryp$^-$ phage, it is possible that the difference between the two phages lies in their relationship to the tryptophan, in which they are inevitably produced, rather than in a demand for tryptophan. The tryp$^+$ phage may have it so loosely combined that it can be detached by dilution. On the other hand the tryp$^-$ phage may hold it in firmer combination and resist deactivation. In this case tryptophan should be equally necessary for the adsorp-

tion of both phages and the series from tryp$^+$ to tryp$^-$ would depend upon the increasing number of tryptophan molecules permanently attached to the tails of the phages, as indifference to added tryptophan increased. The more molecules so attached the greater the likelihood of a fruitful collision between phage and bacterial receptor in the absence of added tryptophan.

If this quantitative difference between the two phages is subject to a genetic control, it would seem that the tryp$^-$ phage is likely to obtain a permanent attachment of tryptophan by means of a gene-controlled synthesis, whereas in the tryp$^+$ direction this character is gradually lost.

In nature, since the phage cannot propagate in the absence of growing bacteria and since growing bacteria are necessarily associated with free amino acids, many of which are adsorption factors, it follows that phage cannot be subjected to the selective pressures used in these experiments and, therefore, that the tryp$^-$ mutant is unlikely to become prominent.

SUMMARY

A bacteriophage is described which is active on a very wide range of *Salmonella typhi* strains. As isolated from natural sources it required tryptophan, in particular, as an adsorption co-factor. Under experimental conditions a series of mutants could be selected which were largely but not entirely indifferent to tryptophan. It is thought probable that the indifference to added tryptophan is due to tryptophan attached to the tail firmly enough to avoid detachment by dilution.

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THE VIRULENCE OF *STAPHYLOCOCCUS PYOGENES* FOR MAN. A STUDY OF THE PROBLEMS OF WOUND INFECTION

S. D. ELEK AND P. E. CONEN

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LITTLE direct evidence is available about the virulence of strains of *Staph. pyogenes* to man. Garrè (1885) infected himself with a strain obtained from a fatal case of osteomyelitis by rubbing a whole slope culture into the skin of the left forearm. Small pustules appeared around the hair follicles within a few hours, which enlarged and eventually coalesced into a large carbuncle, which took three weeks to heal with much scar formation. Similar experiments were carried out subsequently by Bümm (1885) and by Bockhart (1887). In this early work the dosage was not stated with accuracy as the aim was to prove that staphylococci were the true cause of carbuncles and wound infections, rather than an attempt to compare the virulence of different strains. It is noteworthy that both Garrè and Bockhart failed to produce lesions in some of their experiments, and similar failures by other early workers were referred to by Neisser (1928). No quantitative information is available at present on the susceptibility of different individuals and the relative virulence to man of different strains.

The virulence of a microbe always relates to a given animal species. The mere fact that lesions may be set up in one experimental animal gives little if any indication of virulence for another species. In staphylococcal infection, the traditional approach of using a convenient laboratory animal may be completely fallacious, as was shown by the divergent results obtained by Frappier, Sonea and Panisset (1955), when they compared the virulence of a number of strains using different methods and animals. In spite of the vast volume of work done on the toxins of *Staph. pyogenes* there is no clear evidence that the same factors play a part in man, rabbit or guinea-pig. Indeed there is reasonable doubt about the rôle of alpha toxin in man, though this factor undoubtedly plays an important part in the evolution of lesions in rabbits.

As yet we have virtually no information about the minimal infective dose of any pathogen to man : only circumstantial evidence and analogies are available. The advent of penicillin and other antibiotics however, has rendered direct inoculation in volunteers relatively safe. The extensive literature on the virulence of staphylococci at present relates either to the production of coagulase and diffusible toxins demonstrable *in vitro* or *in vivo*, or to experimental lesions in animals, usually rabbits. These methods certainly delineate the group of staphylococci commonly associated with human disease, but they throw no light on possible differences in virulence between strains isolated from different sources. The wide distribution of coagulase-positive staphylococci on the human body and its environment suggests that in the evolution of lesions other factors must be

operative apart from the mere presence of the organisms. Are there differences in the virulence of strains, in the susceptibility of individuals, in the size of the infective dose or in local factors modifying the course of infection ?

The present work was designed to provide an answer to these questions. Since the minimum pus-forming dose of staphylococci for man was not known, we decided to establish this first using a known pyogenic strain. This enabled us to compare different strains and also the susceptibility of different individuals. The actual dose in naturally occurring infections can only be estimated within very wide limits. The rôle of modifying factors is also difficult to assess in man, but some information can be obtained on the basis of the minimum pus-forming dose.

MATERIALS AND METHODS

Staphylococcal strains

Twenty-six coagulase-positive strains of staphylococci were used. Twenty-two were sensitive to penicillin, and the other 4 sensitive to erythromycin, and to at least one other antibiotic. Thirteen strains were obtained from a variety of pyogenic lesions (Nos. 1, 2, 3, 4, 9, 10, 11, 21, 22, 23, 24, 25, 26). All were freshly isolated except a single strain (No. 1) which had been freeze-dried. Thirteen strains were isolated from the anterior nares of healthy individuals (Nos. 5, 6, 7, 8, 12, 13, 14, 15, 16, 17, 18, 19, 20). Unless otherwise stated 18-hr. tryptic digest heart-broth cultures were used and were diluted immediately before use in normal saline to the requisite dosage. Viable counts were made simultaneously with each experiment.

Volunteers

One female and 25 male volunteers participated in the experiments, their ages ranging between 18 and 30. Most subjects joined in one experiment only, but some volunteered for repeated tests. In the earlier experiments a positive control leading to the formation of a pustule was included with the tests in each volunteer, but later experience showed that this was not necessary. The lesions were observed closely for a week and any after-effects were investigated for up to 18 months.

Methods of inoculation

Intradermal or *subcutaneous injections* were made using a tuberculin syringe with an 18-gauge needle, and the required inocula were contained in 0·1 ml.

Superficial scratches were made through a drop of diluted culture with a Hagedorn neede without drawing blood, but so that they remained visible for several days. The drop was wiped off after 1 minute.

Incisions through the full thickness of the skin were made in 4 volunteers with a Bard-Parker " Paragon " No. 15 blade on the left forearm. They were 10 mm. deep and 4 mm. long. Each volunteer received 3 cuts, one with ethyl chloride freezing and two with local " Xylocaine " anaesthesia. One volunteer (A) received a further cut without anaesthesia. When the bleeding had stopped 0·02 ml. of a diluted broth culture was introduced into the wounds by means of an " Agla " micrometer syringe (Burroughs Wellcome Ltd). All 13 wounds were covered with sterile lint held in position with porous plaster and left for 4 days.

Adjuvants

In attempts to enhance infection, the following materials were used :

Mucin.—Two preparations were employed : mucin A was a commercial hog gastric mucin (L. Light and Co. Ltd), and mucin B was Wilson's 1701-W Granular Mucin with a known virulence-enhancement effect. It was given, and kindly tested for enhancement of *Salmonella typhi* in mice, by Dr. H. Smith. Both preparations were made into 5 per cent suspensions in saline and autoclaved at 120° for 15 min.

Starch.—This was a formolized glove powder (K285, Boots) which was autoclaved before use.

Suture material.—Sterile black silk sutures 10 cm. long were soaked in a suitable dilution of an overnight broth culture for 30 min., removed singly, placed on sterile filter paper and dried overnight at 5°. An estimate of the viable organisms carried by each was obtained by shaking 3 sutures separately in a known volume of saline for 20 min. on a Kahn shaker and carrying out a viable count on the supernatant. The sutures were inserted by means of a cutting needle through the entire thickness of the dermis on the anterior aspect of the thigh and covered with dry dressing.

RESULTS

Establishment of the Minimum Pus-Forming Dose and the Rôle of the Route of Inoculation

Intradermal injection

Strains 1, 2, 5, and 6 were injected into volunteers in doses of approximately 20 and 500 organisms. Each dose was injected into at least 2 subjects. Control inoculations of approximately the same numbers of heat-killed organisms were also made. Even the larger inocula produced only erythema, reaching 3 cm. in diameter at 24 hr., and disappearing in 48 hr. These effects were not due to any appreciable bacterial multiplication in the tissues, as the killed controls produced similar effects.

Strain No. 3, recently isolated from a carbuncle, was then tested similarly at higher tenfold steps in 5 volunteers. Results were negative up to the level of 7×10^4. At 7×10^5 one volunteer out of 3 developed a minimal pustule. Only the inocula containing 7 million or more viable cocci consistently induced pus formation and the results are described in detail in Table I. It is known that

700,000

TABLE I.—*Minimum Pus-forming Dose (M.P.D.)*

Pyogenic strain No. 3. Intradermal injection. Reading after 48 hours.

	Dose: $7{\cdot}5 \times 10^6$			Heat-killed control		
Volunteer	Pus	Swelling	Redness	Pus	Swelling	Redness
A	+	+	+++	−	−	+
B	+++	+++	+++	−	−	+
C	++	−	+	−	−	±
D	+++	+++	++++	−	−	++
E	++	+	+	−	−	±

high doses of heat-killed staphylococci may lead to suppuration, but this mechanism could be ruled out, since comparison with the controls indicates that further multiplication of the organisms *in vivo* was necessary to produce this result. This was also supported by a later experiment in which one volunteer was receiving chlortetracycline treatment unknown to us at the time of the experiment. He was given the minimum pus-forming dose of 4 strains with negative results, although the same injections produced pus in another volunteer. This indicates that the bacteriostatic effect of chlortetracycline was enough to prevent pus formation. Some variation occurred in the amount of swelling and redness produced due to the circulating antitoxin level, but this did not affect pus formation to any appreciable extent. It was concluded from these experiments that the minimum pus-forming dose injected in a volume of 0·1 ml. intradermally is about 5 million cocci and that a true infection is established by this dose. This finding was also confirmed in later experiments when 19 volunteers receiving between 2×10^6 and $8{\cdot}5 \times 10^6$ staphylococci intradermally produced lesions

with pus formation, while of 26 receiving between 10^2 and $1{\cdot}5 \times 10^5$ cocci not one produced a pustule.

The unexpectedly large inoculum required to produce a pustule on intradermal injection raised the possibility that this route might be unnatural, and that by reproducing the natural mode of infection a much smaller dose might suffice.

The effect of subcutaneous injection

A freshly isolated pyogenic strain (No. 4) was injected subcutaneously in doses of 10^3 and 10^5 cocci in 0·1 ml. into 2 volunteers (A and M). No lesions were obtained although 5×10^6 organisms of the same strain, injected simultaneously by the intradermal route, produced pus. Further experiments were performed on 5 volunteers using a freshly isolated pyogenic strain (No. 21) and an epidemic strain (No. 22) of phage type 80 in doses of $1{\cdot}6 \times 10^5$ and 10^5 cocci in 0·1 ml. respectively. Again neither strain produced lesions in any of the 5 volunteers. These findings indicate that the subcutaneous minimum pus-forming dose cannot be appreciably less than the intradermal dose.

Infection of full thickness skin incisions

Four volunteers (A, V, W, Y) had 13 wounds inflicted as described. A viable count showed that the inoculum instilled was 2×10^4 organisms (strain No. 21). All the wounds healed by first intention. Not only was there no pus formation, but there was no evidence of inflammation. A dose of 6×10^6 cocci of the same strain injected intradermally into one of the volunteers at the time of the experiment produced a typical lesion with pus.

Infection via scarified skin

Cultures of 2 organisms (Nos. 21, 22) were tested on 3 volunteers. Each drop contained between $5{\cdot}5 \times 10^7$ and $8{\cdot}5 \times 10^7$ per ml. None of the volunteers developed lesions in this experiment, although some positive results were obtained later with higher doses. These will be referred to below.

Application to intact skin

Undiluted 18-hour broth cultures of 5 strains (Nos. 21, 22, 23, 24, 26) were spread over an area of about 1 cm. diameter on the forearms of 2 volunteers. Each strain was placed on both hairy and hairless areas. The drops were left in contact with the skin for a minute and then wiped off. No lesions resulted.

Cultural Factors

The rôle of preformed toxin

The preceding experiments were all done with 18-hour broth cultures diluted to contain the requisite numbers of cocci. This generally represented a dilution in saline of 1 : 40 of the original culture, and it seemed therefore possible that an appreciable amount of preformed toxin was transferred. The gradocol filtrate of the 18-hour tryptic digest heart broth culture of strain No. 3 contained 40 Burnet units of alpha toxin. No further attempt was made to analyse the toxins present beyond testing their effects directly on volunteers. Intradermal injections of 0·1 ml. of 1 : 10 and 1 : 100 dilutions of the filtrate were made into 2 volunteers (A and B). In both, considerable areas of redness, swelling and tenderness developed

with some systemic manifestations. Control broth in the same dilutions was without effect. The 1 : 10 dilution of toxin produced a lesion about 10 cm. in diameter within 24 hr., but no pus formed. The reactions in both volunteers resembled each other closely. The maximal effect occurred at 36 hr. and was associated with malaise, shivering and some fever and diarrhoea. The general effects disappeared a few hours later and the local lesions had entirely cleared within 4 days.

To test the effect of toxin on the infecting dose, washed inocula of the same strain were injected intradermally with excess toxin into 2 volunteers (Table II).

TABLE II.—*Effect of Toxin on M.P.D.*

1 : 10 dil. of 18-hour digest broth filtrate plus washed staphylococci. Strain No. 3. Intradermal injection. Readings at 48 hours.

Volunteer		Washed organism control 4×10^6	Toxin 1 : 10 $+ 7{\cdot}5 \times 10^4$	Toxin 1 : 10 $+ 7{\cdot}5 \times 10^2$
L	Pus	+	−	−
	Swelling	++	+	+
	Redness	++	+	+
M	Pus	+	−	−
	Swelling	+++	++	+
	Redness	+++	+++	++

In both volunteers the washed control inoculum led to pus formation in the same dose as unwashed suspensions, but a smaller number of organisms, even in the presence of excess toxin, failed to set up a pyogenic lesion.

This indicates that preformed toxin has no appreciable potentiating effect. To test whether it plays any part at all further experiments were carried out with a larger number of strains. Four strains from lesions (Nos. 3, 9, 10, 11) and 9 nasal strains (Nos. 12–20) were centrifuged three times in saline to free them as much as possible from preformed toxin. They were injected intradermally in doses of from 2×10^6 to $7{\cdot}5 \times 10^6$ per 0·1 ml. into 8 volunteers (L–S). Each strain was injected into 2 subjects. Six received 4 strains, while 2 received one strain only. These experiments formed part of a larger experiment comparing nasal strains and those from lesions. The results with the washed organisms were positive and not significantly different from those obtained with the same unwashed strains.

The age of the inoculum

It is known in the case of streptococci that during the early phases of growth they are more resistant to phagocytosis than in older cultures. This resistance is by virtue of capsule formation (Hare, 1928, 1929). Furthermore, it has been suggested that staphylococci in the early phases of growth produce a capsule (Lyons, 1937) although this finding has not been confirmed. It seemed of interest therefore to compare the effects of 4-hour and 18-hour broth cultures. Using a freshly isolated pyogenic strain (No. 21) 2 volunteers (A and V) were injected intradermally with $1{\cdot}6 \times 10^3$ and 7×10^4 cocci of a 4-hour culture and with $1{\cdot}3 \times 10^3$ and $4{\cdot}2 \times 10^4$ of an 18-hour culture. No pus was produced and no difference was noted between the 2 sets of injections. This experiment would have

detected a hundredfold potentiation, had the 4-hr.-old cocci been more virulent. In later experiments with superficial scratches, similar results were obtained.

Direct passage from a lesion

It is frequently assumed that an organism in a lesion is more virulent than any subculture. The effect of direct passage from a lesion was difficult to test as the possibility of transferring icterogenic virus had to be considered. An experimental lesion was set up in one volunteer (A) and a suspension made of the pus. After centrifuging lightly, part of the supernatant was injected intradermally into the same volunteer and part used for a viable count. This count showed that the dose inoculated was 10^4 cocci, but no lesion resulted. This again suggests that no gross difference exists between organisms in this state, and those in culture.

Differences Between Strains in Relation to Their Source

The virulence of strains obtained from nasal carriers

The question arises whether all nasal strains are capable of producing wound infection or whether only a minority of them are endowed with this ability. If some of the nasal strains were less virulent, their injection in the same dosage as that producing pus with known pyogenic strains would fail to lead to pustule formation. In the first set of experiments strains from lesions were compared with 4 nasal strains; two of the latter were freshly isolated and the other two were from a freeze-dried collection. Three to four strains were tested on each volunteer simultaneously, care being taken to include at least one pyogenic strain as a control (Table III). Both pyogenic strains caused pus formation in

TABLE III.—*Comparison of Staphylococci from Different Sources.*

Dilutions made from 18-hour cultures in digest broth. Intradermal injection. Readings at 48 hours. + = pus discharging; ± = pus not discharging.

	Pus		Nasal			
Origin / Volunteer	No. 3 $6–7{\cdot}5 \times 10^6$	No. 4 7×10^6	No. 5 $8{\cdot}5 \times 10^6$	No. 6 5×10^6	No. 7 $7{\cdot}5 \times 10^6$	No. 8 $1{\cdot}5 \times 10^6$
A	+	..	+	+	..	..
B	+	..	+	+	..	..
C*	+	..	−	+	..	..
D	+	..	±	±	..	..
E	+	..	+	+	..	..
F	+	+	..	..	+	−
G	+	+	..	..	+	−

* Penicillin after 24 hours.

all volunteers. Three of the nasal strains proved to be as virulent as the pyogenic strains. The fourth failed to produce pus in either of the 2 volunteers. This strain, although coagulase-positive, was deficient in certain respects: it produced delta toxin, but only traces of alpha haemolysin and failed to produce lines with the double gradient method (Elek and Levy, 1950).

In the foregoing experiment dilutions of broth cultures were inoculated. A further experiment comparing nasal strains and those from lesions was carried out, the organisms being washed before inoculation; details of this have been described under the heading of "Cultural factors; the rôle of preformed toxin".

Again, no difference between strains from nasal carriers and those from pus could be found. In this experiment 2 subjects (O and P) gave virtually negative results with all 4 strains tested on them, although the same strains produced pus in the paired volunteer. Subsequent enquiry in the case of one of them (O) showed that throughout the experiment he was receiving antibiotic treatment for another condition. The case of the other negative reactor (P) will be referred to later.

Behaviour of strains obtained from epidemics

Hospital outbreaks of staphylococcal lesions are frequently observed and are generally due to a single phage type. This may be due to increased virulence or to factors leading to increased communicability. Furthermore such strains often produce characteristic lesions in infants which are different from those seen in adults. While we have at present no method of testing increased communicability, the minimum pus-forming dose should throw some light on increased virulence, if that exists.

A strain (No. 22) was freshly isolated from an outbreak of " septic spots " in the maternity department. It also caused some cases of breast abscess and was phage type 80, the prevalent epidemic strain at the time. It was injected intradermally into 4 volunteers in doses of 10^3 and 5×10^4. The volunteers received simultaneously similar doses of a strain obtained from a stye (No. 21). No pus resulted from any of the injections. One volunteer (A) received 6×10^6 of the maternity strain (No. 22) and $1{\cdot}1 \times 10^7$ of the stye strain intradermally. Both strains produced pus but differed in the nature of the lesions. The lesion from the stye strain behaved like previous pustules, but the lesion produced by the maternity strain in the same dose-range was considerably more painful and produced a wider area of surrounding erythema. By the time that the stye strain lesion was regressing the type 80 strain lesion had only just begun to localize. It became a large boil which discharged pus profusely. The volunteer receiving this strain had experienced six previous pustules from various other strains and he commented on the protracted course and extensive nature of this lesion.

The maternity and stye strains were also compared by the subcutaneous injection in 0·1 ml. of 10^5 and $1{\cdot}6 \times 10^5$ cocci respectively into 5 volunteers. All produced negative results.

The natural mode of transmitting pemphigus appears to be through abrasions of the skin. Skin scratches were made on 9 volunteers and covered with lint impregnated with staphylococci. Four type 80 strains isolated from outbreaks in different hospitals (Nos. 22, 23, 24, 25) and the stye strain (No. 21) were compared. A drop containing from 5×10^4 to $1{\cdot}7 \times 10^5$ organisms was placed over the scratch. The lint was held in position for 2 to 4 days with porous plaster. No significant difference could be detected between the stye and the type 80 strains as regards the incidence of lesions. In fact only 6 lesions resulted out of 42 inoculations. However, all the lesions were of a different type from the ones previously observed. They consisted of small blebs on the line of the scratch surrounded by minimal erythema without pain or swelling. These lesions regressed spontaneously within about two days. Scratches were also made through drops of 1 : 10 and undiluted 18-hour broth cultures and the drops wiped off. Again most of the volunteers failed to produce lesions of any kind and no differences berween the strains could be demonstrated.

Thus, using adult volunteers no immediate difference could be shown to exist between epidemic strains and others, although there was a suggestion that the former in the minimum pus-forming dose produced more severe lesions. However, in one volunteer to whose intact skin five undiluted broth cultures were applied without any apparent result, chronic furunculosis appeared 3 weeks later. The staphylococci isolated from the lesions corresponded in their phage type and antibiotic resistance pattern to one of the inoculated strains (No. 22).

The Susceptibility of Individuals

Clinical experience with post-operative wound infection suggests that there is no great variation in the susceptibility of individuals to staphylococcal infection. With outbreaks of this kind a large proportion of people at risk are affected. However, no clear evidence was available. In 17 volunteers a total of 45 inoculation. within the range of the minimum pus-forming doses were made and 40 resulted in some pus formation. Of the 5 failures 3 occurred in the same volunteer (P) who was given 4 strains and reacted with pus formation to only one. Assuming that this volunteer did not receive antibiotic treatment unknown to us, he must be regarded as a more resistant individual. This view is strengthened by the fact that the paired volunteer (Q) receiving the same strains in the same dosage reacted to all 4. The two further instances of failure to react occurred in volunteers (C and S) who produced pus with other strains at the same time. The fault was not in the strains selected, since they were effective in other subjects. This evidence supports the view that the incidence of resistant individuals is low and cannot be an important factor in the general question of staphylococcal wound infection.

There is a popular notion that some individuals are particularly prone to develop staphylococcal lesions. Though chronic furunculosis exists as an entity, it is not known whether such individuals are in fact abnormally susceptible, or whether other factors are responsible for the recurrence of the infection. Two of our volunteers gave a history of boils and styes within the 2 or 3 years preceding the experiments. There was no indication, however, that they reacted in any way differently from the others. Only one individual reacted with a different type of lesion, and then only after scratches. This volunteer (T) was inoculated with 5 strains, including 3 of type 80, by scratching the skin through drops of undiluted 18-hour broth cultures. A saline control and the application of the same material to the intact skin were without effect. All 4 strains produced small blebs in the scarified area with little surrounding redness, pain or swelling. The same treatment in another volunteer (U) was negative. This pemphigus-type reaction may represent a special susceptibility analogous to that seen in very young children.

One of the subjects (A) had repeated inoculations with resulting pus formation over a period of two years, and several other volunteers had at least two challenges within a few weeks. One individual was challenged while recovering from a naturally occurring boil. No evidence of acquired immunity was detected in any of these cases.

The Effect of Added Factors on Pus Formation

It is clearly inconceivable that the very large dose shown to be necessary for experimental infection finds its way into surgical wounds. Other factors must therefore be operative.

The effect of plasma on the minimum pus-forming dose

It seemed possible that intradermal inoculation may not stimulate the outpouring of plasma, which is believed by some to play an integral part in the evolution of natural infection. The rôle of plasma was therefore tested.

Two volunteers were injected intradermally with 0·1 ml. of their own heparinized plasma. Heparin was chosen as anticoagulant because it does not interfere with staphylocoagulase action. Organisms from strain No. 3 (6×10^4 and 6×10^5) were next injected into the wheals and into normal skin. A "positive control" dose of cocci (6×10^6) was also injected into normal skin, and produced typical results in both volunteers, whereas a tenth and a hundredth of this dose with or without plasma failed to produce pus. Thus excess plasma does not produce a demonstrable potentiation of the minimum pus-forming dose. In fact the presence of plasma lessened the redness and swelling stimulated by the lower doses presumably on account of the antitoxin present in normal plasma.

The effect of mucin on the minimum pus-forming dose

In view of the known virulence enhancement effect of certain mucins, and the fact that the natural habitat of staphylococci, the anterior nares, contains such mucin (Nungester, Bosch and Darwin, 1951 ; Smith, 1951), it appeared possible that this mechanism may be operative in natural infections. Two volunteers (J and K) were injected intradermally with 10^4 and 10^5 cocci of strain No. 3 suspended in mucin A. As controls, sterile mucin alone and $5{\cdot}5 \times 10^6$ organisms in saline were also injected intradermally. The organisms in saline produced pus. The mucin alone produced a fairly severe inflammatory reaction without pus formation. The organisms suspended in mucin resulted in lesions similar to mucin alone.

This experiment was repeated with mucin B. Two further volunteers were injected with 5 per cent suspensions of this mucin alone and also with mucin containing $2{\cdot}5 \times 10^2$ and $2{\cdot}5 \times 10^4$ organisms. Even with this preparation (of proven virulence enhancement) no pus was formed, showing that if enhancement exists it is less than 40-fold.

Potentiation by foreign body reaction

The remaining possibility seemed to be that a small infective dose may multiply to the full pus-forming dose under the protection of a foreign body reaction. To test the effect of a foreign body by intradermal injection, a sterile innocuous particulate material is required. Talc and lycopodium seemed unsuitable since they are liable to form granulomas. Formolized starch is used as a substitute for talc glove-powder and can be made into a suspension easy to inject and capable of sterilization. Three volunteers (A, V, W) were injected subcutaneously with a suspension consisting of 6×10^5 starch particles and $1{\cdot}3 \times 10^3$ cocci of strain No. 21 in 0·2 ml. As controls, injections of the particles alone, and organisms alone were made in the same doses. This experiment and another on two further volunteers using 5×10^3 cocci gave entirely negative results.

The frequency of stitch abscesses suggested the possibility that this type of foreign body reaction in some way potentiates infection. Furthermore tight stitches have often been blamed as the cause of suppuration by surgeons, and it seemed possible that the tension might interfere with the phagocytic defence

mechanism. Sutures were infected with strain No. 4, recovered from an experimental lesion in a volunteer. The viable counts showed that the entire length of a suture contained approximately 3×10^4 cocci. Two volunteers (L, M) each received 3 sutures: one stitch was tied with the customary degree of tension, another was tied with an abnormal degree of tightness and the third stitch was pulled right through. Twenty-four hours later both volunteers became very ill and the remaining sutures had to be removed. Where the stitch had been pulled through practically no reaction was visible, although subsequent examination of the suture showed that at least $\frac{4}{5}$ of the inoculum was swept off and left in the tissues. At the other two sites after 48 hours very large stitch abscesses developed. Although according to the volunteers the severity of the lesion at the site of the tight stitches was greater, objectively no clear difference could be found. In spite of penicillin therapy the lesions progressed to the size of an orange, caused fever and other systemic manifestations and took over a week to resolve.

This experiment convincingly demonstrated the enhancing effect of a stitch, but led to great difficulty in finding further volunteers. Another stitch infected with approximately 3×10^2 organisms was inserted into a single volunteer (A) with a sterile stitch as control. Within 24 hours a small stitch abscess exuding pus was produced, even with this small dose, while the sterile stitch showed no reaction. The experiment was discontinued and penicillin treatment instituted at that stage. As the buried part of the stitch contained at the most 10^2 cocci, the result represents a virulence enhancement of the order of at least 10,000 times.

DISCUSSION

The original aim of this work was to find out whether especially virulent strains of staphylococci exist and are more likely to cause wound infection. Experience with outbreaks of sepsis in hospitals indicates that a single phage type is often responsible. It has been tacitly assumed in the past that such a strain possessed higher virulence to man than other coagulase-positive staphylococci carried by hospital staff. While this may be true for impetigo strains, we have been unable to find reports of any work relating directly to the virulence of staphylococci as regards wound infection in man.

The fallacy of simply using the most convenient laboratory animal has been pointed out. There is ample evidence to show that different metabolic products of staphylococci contribute to virulence in different species. Van de Velde (1894) showed that a strain of staphylococci obtained from man could be rendered highly virulent to rabbits by repeated passage, but the latter strain differed in no way from the non-virulent parent strain when tested in dogs. Dold (1927) introduced a form of virulence test in guinea-pigs which consisted of the injection of $2{\cdot}5 \times 10^9$ cocci. This gave a gross differentiation between *Staph. pyogenes* and micrococci but can hardly be described as measuring virulence, since it merely reveals the difference between a pathogen and a non-pathogen. Panton and Valentine (1929) established the minimal pus-forming dose on intradermal injection in rabbits, but did not carry out an extensive comparison between strains. Kourilsky and Mercier in a series of papers (1940, 1941*a*, *b*, *c*, *d*, *e*, *f*, *g*) claimed to have demonstrated differences in virulence between strains obtained from lesions and nasal strains. They also claimed that inflammation had an enhancing effect on virulence, but their technique allowed the carry-over of preformed toxin and their endpoint was not

pus formation. Many other comparisons are available on rabbits. When death is taken as the endpoint, the presence or absence of alpha toxin production is measured. There is no clear evidence that alpha haemolysin enters into the evolution of staphylococcal lesions in man, and if it does, it is clearly not of decisive importance, as is shown by the failure of antitoxin treatment. The same considerations apply to the lethal effect in mice, and to the measurement of the size of lesions produced, both of which show correlation with the amount of alpha lysin production (Christie, North and Parkin, 1946 ; Gorrill, 1951 ; Selbie and Simon, 1952). More recently Frappier and Sonea (1953, 1954) by subcutaneous injection of washed staphylococci into 18- to 19-day-old chick embryos claimed to have shown great differences in their virulence. With some strains a 50 per cent mortality could be obtained with less than ten cocci. In this experiment the rôle of preformed toxin was excluded, but it was assumed that the chick embryo defences react like those of man—a quite unwarranted assumption. Furthermore adult birds are known to be highly resistant to human staphylococci, but susceptible to bird-pathogenic strains. It seems to us therefore that no reliable information can be deduced about the virulence of staphylococci to man from experiments carried out solely on animals.

Experiments on human volunteers are restricted by their very nature. Within these limitations no gross differences could be demonstrated between strains from pyogenic lesions and from nasal carriers. It is possible that had the comparison been carried out at all levels, rather than only at the minimal pus-forming level of known virulent strains, some slight differences might have been observed. These, even if they existed, must be insignificant however compared with the enhancing effect shown by a foreign body.

The striking similarity in the evolution of the lesions between different volunteers suggests that at the minimal pus-forming level no great variation in susceptibility exists. Only in one subject could we find any indication of increased resistance. If pus formation is taken as the criterion, neither preformed toxin, nor the presence of excess plasma alters the findings. Toxin by itself failed to produce pus, even in doses high enough to cause severe and extensive local reaction and systemic disturbance.

Clinical evidence suggests that staphylococcal lesions represents essentially a local battle. When the local defences are overcome, extension occurs and a fresh battle is fought out at an adjacent or distant site. Even the most advanced pyaemia must be regarded as a summation of these events.

Little is known about the factors that destroy coagulase-positive staphylococci in lesions. Spink and Vivino (1942) showed that they survive the action of whole human blood and Rogers and Tompsett (1952) confirmed that pyogenic staphylococci survive even after ingestion by leucocytes. Long (1927) had already observed these effects and in addition demonstrated that inflammatory exudates previously induced in animals by chemical means exert a sterilising effect on staphylococci. That such factors operate is shown by the spontaneous healing of a boil.

The number of cocci present may be a factor influencing the end-results. Young (1954) showed that injected staphylococci disseminated rapidly in the cheek pouch of hamsters, but an abscess developed only at the site of inoculation, where the inoculum density was highest. Our findings show that a critical minimal concentration of cocci is required in man to establish a pustule. This critical dose is very high—apparently more than one million cocci per 0·1 ml. on intradermal

injection and not appreciably less for other forms of inoculation. Infection of this magnitude is unlikely to occur under natural conditions since a nasal droplet of 100 microns diameter could not accommodate this number, even if it consisted entirely of staphylococci. However, the critical concentration of cocci may be reached from a smaller dose if the defence mechanism is delayed.

Such delay may be produced by a variety of agents and the potentiation of staphylococcal lesions in experimental animals may be dramatic (Evans, Miles and Niven 1948; Miles, Miles and Burke 1957). These agents are believed to inhibit the access of bactericidal elements of the blood to the lesion. This failure of mobilization is temporary. lasting ouly for a few hours. It is likely that soft tissue trauma (Pazzagli and Zambelli, 1934) and burns also give a temporary advantage to the inoculum, thus permitting a smaller infective dose. The best-known general enhancing agent is the factor in hog gastric mucin. Our failure to demonstrate appreciable enhancement with it suggests that the mucin of nasal droplets is not a factor in the initiation of wound infection. The enhancement obtained by sutures however is so tremendous that the foreign body reaction must play a major part in wound infection. Clearly any delay in the as yet unknown sterilizing mechanism may convert an otherwise insufficient dose of infection into the minimal pus-forming dose. Such delay need not be very great in terms of time. With a generation time of 30 min. only 7 hr. of logarithmic growth would be required to convert a hundred cocci into over a million. This mechanistic view may not be the true explanation and it is possible that the foreign body reaction in some way interferes with the sterilization of the cocci. There is other evidence in favour of the concept that the formation of a microcolony is required to cause a pustule. Bockhart showed in 1887 that rubbing a culture into the hairy skin sets up multiple small pustules mostly around hair follicles. Sections taken from such lesions indicated that the organisms multiplied in the sweat glands and hair follicles. Presumably when a microcolony by virtue of its size and toxin production sufficiently damages the epithelium to burst through, a larger lesion develops. This concept is in agreement with clinical experience in furunculosis.

The evidence presented here suggests that much of the work on the virulence of staphylococci may have to be re-examined. The puzzling feature of staphylococcal wound infection is not why it arises, but why it does not occur more often. In view of the wide dissemination of this pathogen it is unlikely that wounds can be kept entirely free of contamination by *Staph. pyogenes* even when strict surgical asepsis is practised. Duguid and Wallace (1948) estimated that with the average activity of daily life the air becomes contaminated from personal clothing at the rate of about one million bacteria-carrying dust particles per person per day. The usual sterile uniform worn in the operating theatre reduced the count by only half. With nasal carriers coagulase-positive staphylococci were recovered from 10 cu. ft. of air during activity and for 10 min. afterwards—in spite of the wearing of masks over mouth and nose. Clearly the great majority of contaminated wounds fail to suppurate. Our experimental findings are entirely in agreement with this observation. The magnitude of the minimum pus-forming dose shows that man is highly resistant to *Staph. pyogenes* and special circumstances are required to enable the ordinary infective dose to reach the minimal pus-forming level. The presence of a foreign body, such as a suture, may provide these conditions. Other circumstances which may lead to the unhindered growth of small inocula include heavily traumatized tissues, burns or devitalized tissues distal to the

ligated vessels. This may be the explanation of the traditional surgical view that untidy operation techniques predispose to infection.

In naturally-occurring skin lesions small differences in virulence may be far less important than the unexplained mechanism whereby secretions in sweat glands and hair follicles prevent the free growth of staphylococci. That these in turn are related to endocrine function is suggested by the frequency of boils at puberty. It is noteworthy that although staphylococci may be incapable of setting up lesions in guinea-pigs normally, a large abscess is produced if the same dose in injected intradermally in lanoline and oil (Lagrange and Lagrange, 1935).

Insufficient work has been carried out with impetigo strains to assess their virulence in relation to others. Their undoubted epidemicity indicates that they differ in some respect. This may be a small increase in virulence. A large increase in virulence seems unlikely on the evidence of our experiments. Undue communicability may well be responsible for outbreaks, but no method to measure it exists. The difference may be in the special host-parasite relationship between staphylococci and the skin of infants. A hint that such special susceptibility to an unknown factor may exist was given by one volunteer who reacted to infected scratches with pemphigus-like lesions.

It must be borne in mind that the surface barriers of the body are in all probability constantly being breached by staphylococci. Their numbers in man's environment are so great and minor injuries so frequent that they must reach the tissues very often, but the efficiency of the defensive mechanism is such that disease is uncommon. When within a given space they reach a critical number, the balance turns in their favour. As gross variations in virulence do not appear to exist for man, the circumstances of the infection rather than the infective dose determine the outcome.

SUMMARY

The minimum pus-forming dose of *Staphylococcus pyogenes* on intradermal injection into human volunteers was established. With strains freshly isolated from lesions, no pus formation was obtained with less than a million cocci in a volume of 0·1 ml. It was estimated that the minimum pus-forming dose for virulent staphylococci was of the order of 2 to 8 million organisms.

Subcutaneous injection and infection of full thickness cuts in the skin, showed that the minimum pus-forming dose by these routes was no less.

No difference in the minimum pus-forming dose was demonstrable between strains obtained from lesions, from healthy nasal carriers and epidemic strains.

The presence of excess staphylococcal toxin, mucin, human plasma or starch failed to reduce the minimum pus-forming dose. The physiological age of the organisms had no appreciable effect.

The presence of a foreign body reaction in the form of sutures however resulted in a dramatic reduction of the minimum inoculum required to produce pus.

Natural infection with the full minimum pus-forming dose is highly improbable. It is suggested that factors delaying the normal defence mechanism allow the critical pus-forming dose to be reached, and that in wound infections the circumstances of the infection play the major part in the development of clinical lesions.

We wish to express our sincere thanks to our colleagues and to those students of St. George's Hospital Medical School who volunteered for these experiments.

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TRANSFORMATION IN TISSUE CULTURE CELL LINES: THE POSSIBLE GENETIC MECHANISM

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OF the many problems associated with the serial cultivation of cells derived from normal mammalian tissues, none is more challenging or is receiving more attention than that of cell "transformation" and its relation to malignancy. Many of the cell lines now available have been established as a result of this change occurring early in their culture history and leading to the sudden emergence of a stable, rapidly multiplying, epithelial cell type easily handled in culture. The common experience of many workers has now established that the early cultivation of cells derived from normal mammalian tissues usually follows a characteristic course. The lag period which follows the seeding of the original cell suspension is succeeded by a period of active proliferation which is usually sufficiently prolonged to allow of several transplant generations. This phase is followed by a slowing of multiplication to a point where it may cease altogether and at which the original cell type is almost invariably lost. The survival of the cell line then depends on the occurrence of transformation, with the emergence of the new epithelial cell type. Transformation never occurs until this critical period of slow multiplication is reached, and we know of only one instance, that of the monkey kidney line considered below, in which the parent cell type has been successfully continued after the occurrence of transformation.

Transformed cell lines have considerable practical importance in virus studies and the fact that one such line, derived from the kidney of an embryo rabbit, will support the multiplication to high titre of all types of poliomyelitis virus opens up the possibility of virus vaccine production in serially cultivated cells of non-primate origin (Sheffield and Churcher, 1957; Westwood, Macpherson and Titmuss, 1957). It is therefore of the greatest practical importance that the mechanism of transformation and its relationship to malignant change should be understood.

Studies of malignant potentiality *in vivo* are complicated by the phenomenon of immunity to heterografts. Since measures taken to eliminate such immunity, as, for instance, irradiation and cortisone treatment (Moore, Southam and Sternberg, 1956) will permit the establishment of normal tissues taken straight from the body of an alien species (Toolan, 1951; Ford, Hamerton, Barnes and Loutit, 1956) there is great difficulty in the interpretation of results obtained in animals so treated. It may be that the recent work of Foley and Handler (1957) in hamsters will provide the answer to this particular problem. An alternative approach lies in attaining a full understanding of the *in vitro* process and its relation to those *in vivo* changes which lead to malignancy. The only direct evidence on possible

mechanisms so far published is that of Hsu and Moorhead (1957) who base their hypothesis on the demonstrable polyploidy of transformed cell lines.

The work reported here was undertaken specifically to investigate the mechanism of transformation and, while we fully confirm the results of Hsu and Moorhead, we are not in complete agreement with their interpretation.

When this work was started the choice appeared to lie between three possible mechanisms :

(*a*) Adaptation, which may be defined as alteration of the phenotype by cytoplasmic modifications not involving modification of the genotype.

(*b*) Mutation, here defined as alteration of the genotype by any one of a number of different mechanisms (Darlington and Mather, 1949).

(*c*) The emergence of a cell type already present, but unrecognised, in primary culture.

Reasons were given by Westwood *et al.* (1957) for supposing that the transformed cells did in fact represent a new cell type, and the sudden nature of the change, involving few cells and leading at a single step to a new and stable cell type, led them to eliminate adaptation and suggest mutation as the likely mechanism.

The work to be reported has shown conclusively that so simple a hypothesis is quite untenable and evidence will be presented on which a new hypothesis may be based.

MATERIALS AND METHODS

Tissue cell lines.—The origin and isolation of all but one of the cell lines discussed in this paper have been previously described in detail (Westwood *et. al.* 1957). The new line, ERK2, was derived from the kidney tissue of an embryo rabbit and will be described in the text.

Medium.—The basic medium used for the isolation and cultivation of all cell lines consisted of 10 or 15 per cent inactivated serum as specified in the text, 5 per cent tryptic meat broth, 0·5 per cent yeast extract, 0·1 per cent Fildes' peptic digest of sheeps' blood, 0·02 per cent cysteine hydrochloride, and Earle's saline to 100 per cent. In certain instances 0·5 per cent lactalbumin hydrolysate was added to the standard medium. Penicillin 100 units/ml. was added to all media. Streptomycin 100 micro g./ml. and mycostatin up to 300 micro g./ml. were sometimes added.

Culture methods.—Cells were cultivated directly on glass, suspensions for both primary culture and subculture being prepared by means of 0·01 per cent Armour crystalline trypsin, 0·25 per cent Difco Trypsin 1/250, or 0·05 per cent EDTA (Ethylene-diamine-tetra-acetic acid. Disodium salt. British Drug Houses, Ltd.). Routine cultures and most experiments were carried out in 8 cm. Carrel flasks, but for microscopic examination cells were cultivated in slide cells of the type described by Westwood *et. al.* (1957).

Chromosome counts were performed by a technique developed from that of Ford *et. al.* (1956) and Levan (1956). Two-thirds confluent Carrel flask cultures were treated with one in thirty million Colcemid (Ciba) in medium for periods varying from 6 hr. for the fast-growing cells to 18 hr. for the slow-growing. The medium was then removed, the culture washed with phosphate-buffered saline and the cells stripped from the glass with 0·05 per cent EDTA. The cells were deposited by light centrifugation, resuspended gently in 0·7 per cent sodium citrate solution and left for one hour before being again deposited by light centrifugation in a conical centrifuge tube. The supernatant was discarded and the cells resuspended in the fluid residue by very gently flicking the bottom of the tube. Two ml. of acetocarmine were then added drop by drop with gentle shaking and the cells left overnight, by which time they had hardened sufficiently to withstand squashing. Shortening and separation of chromosomes by this technique was excellent though staining tended to be weak.

EXPERIMENTAL RESULTS

Behaviour of Monkey Kidney Line, MK3

During the course of cultivation through 60 transplant generations in 450 days, the cell line MK3 underwent fluctuations in morphology and behaviour which may best be appreciated by comparison with the untransformed monkey kidney line MK1 and the transformed line MK2.

Cell lines MK1 and MK2

The line MK1 was derived from the cortical kidney tissue of a 2·7 kg. rhesus monkey and has been cultivated throughout in standard medium containing 15 per cent calf serum. It underwent transformation in two out of four cultures at the 4th passage and gave rise to the transformed line MK2. The orginal cell type was successfully carried on from the unchanged cultures as the line MK1 now in its 62nd passage after 436 days. The MK1 line and the typical transformed line MK2 are thus derived from the same kidney and spring from the same tissue culture stem.

The similarity in the pattern of growth of MK1 to primary culture is shown in Fig. 2 and 3. In Fig. 4 is shown a culture of MK1 at an earlier stage of development and it can be seen that the early growth consists of tongues of mutually oriented cells which unite to form a lattice, the spaces of which are later filled by cells frequently arranged in the fan-like manner shown in Fig. 3. As a result of this oriented growth, the final cell sheet shows an elementary structure resembling that of primary culture and quite distinct from the unoriented growth characteristic of MK2 and the other transformed cell lines (Fig. 5). The difference in cellular morphology is clearly shown at higher power in Fig. 6 and 7.

These distinctions of morphology and arrangement between the untransformed cells of MK1 and the transformed cells of MK2 serve as the model against which the changing behaviour of MK3 may be assessed.

Cell line MK3

Establishment and subsequent behaviour.—The line MK3 was established following transformation occurring at the 4th passage of cells derived from the cortical kidney tissue of a 2·7 kg. rhesus monkey. Primary cultures were started in standard medium containing 10 per cent of autologous serum but were transferred to a medium containing 10 per cent of a mixture of calf and human sera. At the 9th passage the cells were again transferred to a new medium containing 15 per cent of calf serum and 0·5 per cent lactalbumin hydrolysate in addition to the standard supplement. This final medium has been used for all subsequent cultivation.

Immediately following transformation the morphology and pattern of growth of MK3 closely resembled those of MK2, though the cells were somewhat larger (Fig. 7 and 8). No change in morphology occurred until the 12th passage, but from then on a slight tendency to pleomorphism was noted, the cells tending to be elongated and variable in size. By the 24th passage the cells were uniformly elongated and during the next three passages their morphology and behaviour reverted rapidly towards the original MK1 type. The completeness of the change

may be judged from a comparison of Fig. 14 with Fig. 2, 3 and 5. Fig. 9 shows a high power view of the cells of MK3 at this stage and the resemblance of their individual morphology to that of the cells of MK1 (Fig. 6) is obvious.

The change in morphology and behaviour was accompanied during the last three passages by a marked slowing of the multiplication rate, but in the 27th passage, when multiplication had almost ceased, a second episode of transformation took place with the appearance of typical plaques of transformed cells in each of three cultures. Following this second episode multiplication was again rapid and, though a tendency to elongation was always present, transformed morphology was maintained until about the 40th passage. At this time more general elongation again supervened though the reversion was not as complete as that seen in the 26th passage. In the 44th passage transformation occurred for the third time, dense, heaped up colonies of cells appearing. Although this third episode was not closely followed its occurrence indicated that the cells were still in an unstable state and even in its 62nd passage the line still shows a tendency to pleomorphism.

Effect of reversion on multiplication rate.—During the routine subculture of the cell line it was noted that the multiplication rate slowed markedly during the last three passages before the second transformation took place. Although quantitative records of the multiplication rate were not kept during routine maintenance, the cell line was used, between its 9th and 43rd passages, in a series of growth curves for the comparison of different media. In the table are shown the mean doubling times obtained in standard control medium from all the experiments in which these cells were used. It can be seen that there was considerable variation in the doubling times obtained at the various passages and, if unknown extraneous factors were not responsible for these variations, there would appear to have been two periods of slow growth, at the 12th and 26th passages. The first of these periods was not noticed during routine passage, but the second corresponds with the passage before transformation took place and fits well with the qualitative observations.

TABLE.—*Effect of Reversion on Multiplication Rate of MK3*

Experiment	Passage	Mean doubling time in hours
1	9	37
2	12	72
3	12	64
4	13	32
5	24	32
6	26	80
7	43	37

Conclusions.—Since morphology and behaviour in culture are the only two markers at present available for the determination of cell type, the use of the term " reversion " for describing the drift back towards untransformed type may be open to criticism, but the pattern of change suggests that this is a not unreasonable interpretation. The implication is that there is no hard and fast barrier between the two cell types as would be expected if the original transformation had been due to simple mutation, and the thrice repeated cycle of events heavily underlines this interpretation. The reversion could not have been due to a back-mutation since it was a gradual process affecting the entire culture and would, moreover,

have necessitated the replacement of a fast-growing cell type by one growing at only half the rate. If, however, mutation were eliminated as the basis of transformation the stable difference between the lines MK1 and MK2, which sprang from the same tissue culture stem, would become difficult to understand. This difference most strongly suggests a genetic basis and the apparent contradiction is emphasised by the pseudo-transformation which may be caused by environmental factors and which is considered in the next section.

Attempts to Induce Transformation

Influence of the environment

In an attempt to study the influence of environmental factors in inducing transformation, a new cell line was started from the kidneys of an embryo rabbit of about 26 days gestation. The cells were cultivated initially in standard calf serum medium with the addition of 0·5 per cent lactalbumin hydrolysate, and the maintenance line was carried exclusively in this medium. Subcultures were made, usually from one into two flasks, when cultures became densely confluent.

During the first fifteen passages the cell line was closely watched for signs of slowing of the multiplication rate and the appearance of cells of transformed type, but no such changes occurred and as early as the 10th passage the cells appeared to be well established. In primary culture considerable diversity of cell type was, as usual, seen, but this diversity was rapidly lost and by the 6th passage the cells had attained a uniform spindle cell morphology (Fig. 10). The line is now in its 47th passage after 253 days of cultivation and is still in the untransformed state.

From the 4th passage onwards various modifications were made in the environmental conditions of test cultures in an unsuccessful attempt to induce transformation, and one of the experiments will be described in detail since it demonstrates a very important effect which the environment may exert on morphology and behaviour.

It has long been known that a certain minimum cell density, relative to the volume of medium present, is required before cells will multiply in culture, and it seemed possible that, if the critical cell density could be attained at which the cells would just survive without multiplication, the condition of very slow or absent multiplication at the time of transformation might be reproduced. Dilutions of inoculum were therefore prepared to contain 8, 4, 2 and 1×10^5 cells in 10 ml. of medium and 10 ml. quantities were inoculated to Carrel flasks. The results of one such experiment carried out at the 7th passage are shown in Fig. 10–13. At a density of 4×10^5 cells and above per flask the cells multiplied rapidly to produce a dense spindle-cell sheet of uniform cell type (Fig. 10). This was the type of growth obtained in the routine subcultures already mentioned. With 2×10^5 cells per flask multiplication was not quite so rapid and differentiation occurred into two distinct cell types, " epithelial cell " plaques being separated by intervening cells of " fibroblastic " form (Fig. 11). The cells of the plaques, though suggestively epithelial in morphology, tended towards the spindle form and contained large granules in their cytoplasm (Fig. 15). Epithelial cells of this type and containing similar granules had been previously noted in primary, 3rd passage and 6th passage cultures, and could still be obtained at the 46th passage by suitable adjustment of conditions. In cultures inoculated with 10^5 cells the critical inoculum was attained and the cells did not multiply. They spread out

over the glass surface to give the straggly appearance shown by many of the cells in Fig. 12. Such a culture encountered during routine passage would be considered to have no hope of survival. The cells did not, however, die, but survived in this condition for about 14 days before any change was noted. At the end of this time a few small compact cells were observed which showed no tendency to spread over the surface of the glass. At their first appearance these cells were thinly scattered, singly or in small groups, throughout the culture (Fig. 12), but rapidly multiplied to form small colonies of cells (Fig. 13). As the colonies, or plaques, developed many of the cells remained rounded, though attached to the glass, but some spread to form typical epithelial cells of polygonal shape. When plaques of this type were first observed they were thought to be typical of the transformation process, but careful observation of the cells showed that they had the characteristic granulation previously noted, and which we have never observed in transformed cells, and further, as the population density increased they tended to become progressively more spindle-shaped until they closely resembled those seen at the next higher cell concentration. The straggly cells which covered the intervening areas showed no signs of recovery from their unhealthy condition even after numerous plaques had reached a diameter of 1 cm. or more. When subculture was carried out from such a culture, the plaques being pipetted off the glass with the aid of trypsin and the inocula being again diluted in series, precisely the same pattern of events was repeated at the same cell concentrations. In one experiment four consecutive series of subcultures were carried out, the inoculum at each step being obtained from plaques developing in the most dilute culture which produced multiplication in the previous series. At each step it was found that the critical inoculum was approximately 10^5 cells, or 2×10^3 cells per cm.^{2} of glass surface, and that the pattern of behaviour remained unaltered throughout the experiment. High density inocula always led to the formation of a uniform spindle-cell sheet, while at the critical inoculum epithelial-cell plaques were eventually formed.

Conclusion.—The influence of variations in the environment on cell morphology is well known, as for instance the effect of different types of sera on the morphology of transformed cells noted by Puck, Marcus and Cieciura (1956) and Westwood *et al.* (1957) but these instances represent the influence of the macro-environment on the cells of the entire culture. The significance of the present observations lies in the fact that cells which have been reduced to apparently uniform morphology by *in vitro* passage can still be induced to differentiate into " fibroblastic " and " epithelial " types by variations in the micro-environment governed solely by the proximity of other cells. Moreover the epithelial morphology during early plaque formation, which so closely resembled that of transformed cells, was clearly not heritable but fitted well the concept of an " adaptation "—a true cytoplasmic response to local environmental conditions—thereby stressing the probable genetic basis for the stable, heritable differences between MK1 and MK2. The contradictions presented by the complex events described are resolvable only when they are considered in conjunction with what is known of the mechanisms of gene action and the chromosome patterns of cells in culture. As Hsu and Moorhead (1957) have pointed out, there is now much evidence, derived from their own work and that of other workers (Moore *et al.*, 1956 ; Leslie, Fulton and Sinclair, 1956 ; Levan, 1956 ; Westwood *et al.* 1957), that transformed cell lines are polyploid no matter what their origin. Moreover, Hsu and Pomerat (1953) showed that although the overwhelming majority of cells from normal mammalian

tissue is diploid, there is always a small proportion of aneuploids and an even smaller proportion of polyploids. If transformation depended on the presence of these few polyploid cells then its occurrence in a few cells only of a culture would be readily explained. This is the mechanism favoured by Hsu and Moorhead (1957) who suggest that the loss of vigour of the diploid stock after a series of divisions *in vitro* permits the emergence of the polyploid cells which, by virtue of their genotypic diversity, may be better able to adapt to tissue culture conditions. This explanation is faced with two difficulties; it explains neither the loss of vigour of the original cell type nor why the adaptation of the polyploid cells should be uniformly delayed until that loss of vigour has taken place. The first of these difficulties will be considered in a later section, but the second might be explained by a possibility arising from the findings of Hsu and Pomerat (1953). These authors suggest that the pattern of chromosome distribution is constant in any one tissue of any one species and a similar constancy is found in the cells of ascites tumours where it has given rise to the stem-line hypothesis. It has been generally assumed that the polyploid cells found in small numbers in normal tissues are incapable of sustained multiplication as a consequence of genetic imbalance, but it is also possible that their failure to multiply is due to a positive suppressive action exerted by the normal diploid population. If such suppressive action were carried over into *in vitro* cultivation the dormancy of the polyploid cells, until the diploids had lost their vigour, would be understandable. The direct testing of such a hypothesis is not easy but the experiments to be described in the next section were designed to this end.

Attempt to Demonstrate Antagonism between Cell Types

Primary cultures of embryo rabbit kidney and two established cell lines, also derived from embryo rabbit kidneys, were used for these experiments. The origin of the transformed line ERK1 has been previously described (Westwood *et al.*, 1957) and the line is now in its 137th passage after 766 days of cultivation. The untransformed line ERK2 originated as already described (page 591).

In the first experiment suspensions of ERK1 and ERK2 cells were prepared and ten drops of each, containing about 10^3 cells per drop, were distributed in dry Carrel flasks so as to form ten discrete colonies. Separate flasks were used for each cell line. The flasks were incubated for 24 hours and then 5 ml. of medium carefully added so as not to disturb the attached cells. Six hours later a further 4 ml. of medium, containing approximately 10^6 cells of the opposite type was added to each culture. During the next three days it became obvious that, so far from antagonism, a synergic effect was produced, each cell type multiplying more rapidly immediately around and amongst the cells of the opposite type. It was thought that the apparent synergism was probably due to the higher cell density in the regions where the opposite cell type was present.

As will be seen later, the cells of ERK2, at this time in their 20th passage, were already showing a considerable degree of polyploidy, so the experiment, though demonstrating lack of antagonism between the transformed and untransformed cells, has little bearing on the possible behaviour of normal cells. The experiment was therefore repeated using both the ERK1 and ERK2 lines in combination with primary cell suspensions prepared from embryo rabbit kidneys. Precisely the same result was obtained, synergism rather than antagonism being observed with all combinations.

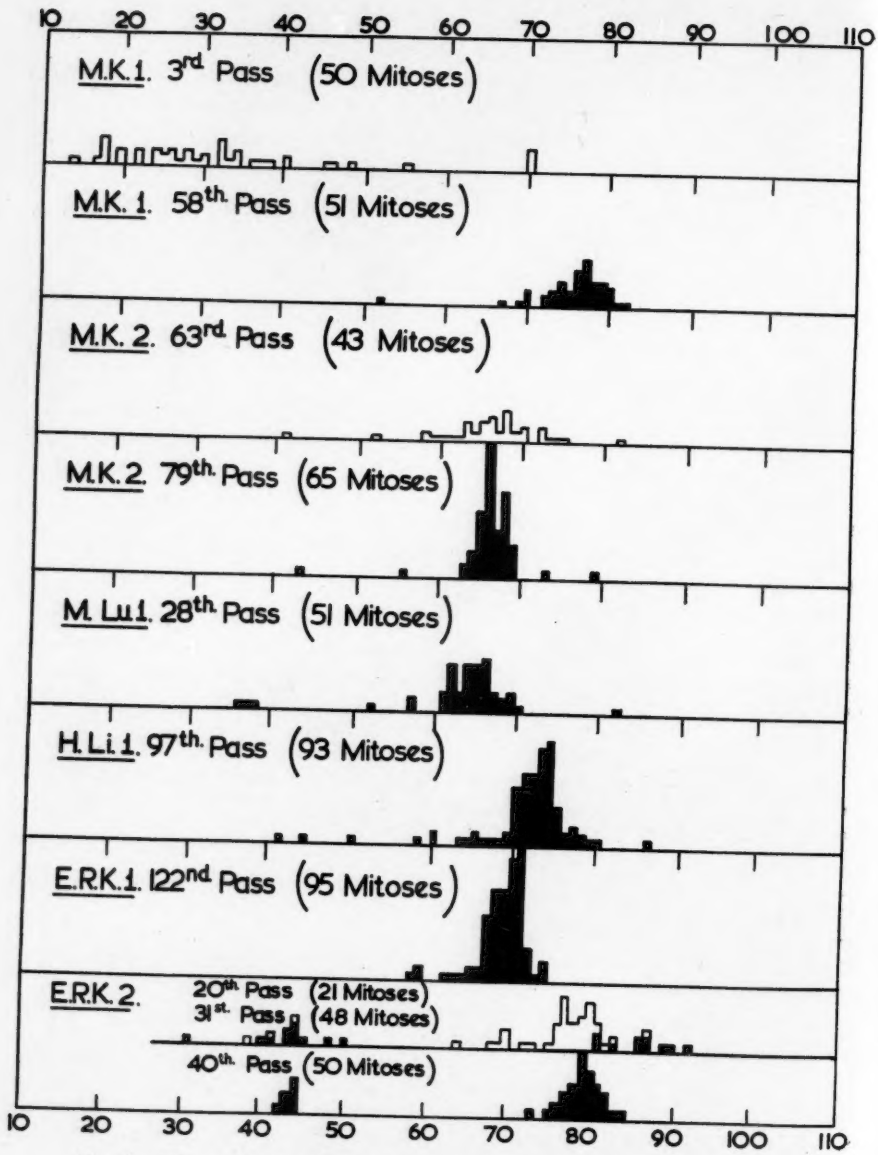
10 20 30 40 50 60 70 80 90 100 110
M.K.1. 3rd. Pass (50 Mitoses)
M.K. 1. 58th. Pass (51 Mitoses)
M.K. 2. 63rd. Pass (43 Mitoses)
M.K.2. 79th. Pass (65 Mitoses)
M. Lu 1. 28th. Pass (51 Mitoses)
H. Li 1. 97th. Pass (93 Mitoses)
E.R.K.1. 122nd. Pass (95 Mitoses)
E.R.K. 2.
20th. Pass (21 Mitoses)
31st. Pass (48 Mitoses)
40th. Pass (50 Mitoses)
10 20 30 40 50 60 70 80 90 100 110

FIG. 1.—MK = monkey kidney; M.Lu = monkey lung; H.Li = human liver; ERK = embryo rabbit kidney.

This second experiment was not performed until the ERK2 line was in its 35th passage by which time over 80 per cent of its cells were in the polyploid state. It therefore demonstrates that diploid cells in primary culture do not suppress the multiplication of polyploid cells as such, whether these be transformed or untransformed, but it is clearly possible the established cell lines have, after their long sojourn *in vitro*, become impervious to some controlling factor to which polyploid cells *in vivo* might still be subject. Although, therefore, these experiments cannot be considered conclusive, the failure to demonstrate antagonism leaves unanswered the second objection to the hypothesis advanced by Hsu and Moorhead (1957).

Chromosome Patterns

In Fig. 1 are presented a series of histograms constructed from chromosome counts on the cell lines under study in this laboratory.

Except in the case of the 20th passage of ERK2, the solid histograms represent exact counts on from 50 to 100 mitoses, no mitoses being included on which an exact count could not be made with confidence. While this procedure demands a more rigid selection than is usually exercised, it is not thought to bias the distribution of counts since the successful separation of chromosomes by the technique used depends on the stage of mitosis rather than on the total number of chromosomes. Thus comparison of the two histograms for MK2 shows that though the inaccurate counts have a wider spread, as might be expected, the mode is the same in each case. The open histograms, and that for the 20th passage of ERK2, are from counts made before the technique was fully worked out, and carry an error of about $\pm$ 3 in the tetraploid region, though most of the diploid figures are exact. In all lines a small number of cells in the octoploid region were counted, but these did not affect the comparisons to be discussed and have been omitted for the sake of simplicity.

In claiming exact chromosome counts on these cell lines one caution is necessary. In cells of MK1 up to three minute chromosomes were usually present (Fig. 16), and these were so small that no matter how good the spreading achieved it was impossible to be sure that none had been masked. Chromosomes of this order of size were less frequently seen in the other cell lines, but even here the possibility must be recognised.

When the counts presented in Fig. 1 are compared with those of Hsu and Moorhead (1957) there is a very strong suggestion that all established cell lines will be found to be polyploid, whether they are transformed or not, and all those so far examined fall into the " hypotetraploid " region. There is, moreover, a much stronger suggestion of a stem-line region in our results than in any hitherto published. This is almost certainly due to the precision of the technique and may be of practical importance in the positive identification of cell lines. For instance the line ERK1, which is of particular interest for its ability to support the multiplication of poliomyelitis virus, is clearly distinguishable from any of the other transformed lines in our series. It will be necessary, however, to determine how much variation may occur in the chromosome distribution from passage to passage though the modal similarity in the 63rd and 79th passages of MK2 suggests that the distribution may in fact remain constant.

Of greater theoretical interest, however, are the counts from the untransformed lines MK1 and ERK2 for in them can be discerned a pattern which may provide

the key to an understanding of the changes, including transformation, which occur during early cultivation. Firstly it is clear that polyploidy is as characteristic of untransformed as of transformed cells, hence its acquisition cannot itself be the basic mechanism of transformation. Secondly, in the third passage of MK1, the passage before that in which transformation of these cells occurred, the chromosome numbers had fallen far below the diploid figure, and this observation suggests the mechanism by which the loss of vigour of the original diploid stock is brought about. The genetic constitution of mammalian cells is highly complex, and much of the genetic material concerned with hereditary mechanisms must be surplus to the requirements of life *in vitro* where the basic necessity is the possession of a complete metabolic system able to utilise those raw materials supplied in the medium. Loss of chromosomes through aberrant mitosis might, therefore, be tolerated so long as important metabolic pathways were not impaired. Yet a limit must ultimately be reached beyond which cells are either unable to maintain themselves or unable to divide, and the line must then die out unless some cells remain which have preserved their metabolic systems intact, and it is here that the possible importance of the polyploid population arises. As was pointed out by Hsu and Moorhead (1957) such cells, by virtue of their greater genotypic diversity, might be expected to have greater adaptive capacity, and there is no obvious reason why the presence of irrelevant genetic material should impair their ability to survive. Hsu and Moorhead favour the suggestion that adaptation to *in vitro* conditions depends on the environmental selection of the polyploid population, and the chromosome changes seen in MK1 clearly shows how such selection might operate. Yet the behaviour of ERK2 does not support this hypothesis. Early chromosome studies were not made on this line, but it may be seen that as late as the 20th passage, 10 passages and 52 days after the line was judged to be "established", half the cells were still in the diploid state, the rest being tetraploid. Since a full diploid set of chromosomes meets the metabolic requirements of cells in culture, as may be inferred from the success of primary culture and early subculture, this fact in itself need occasion no surprise, but it does imply that this line did not suffer gross mitotic aberrations during early passage. By the 31st passage over 80 per cent of the cells had become tetraploid without there being any apparent increase in the degree of adaptation or any change in morphology or behaviour. In this instance, therefore, full adaptation would appear to have occurred in the diploid state with the acquisition of polyploidy as a purely secondary phenomenon of no obvious advantage or disadvantage to the cells. One of the many possible mechanisms by which the change may have been brought about is suggested by the mitosis shown in Fig. 17 which gives clear evidence of endoreduplication (Levan and Hauschka, 1953) in a cell with, originally, 74 chromosomes. Such mitoses have not yet been encountered in ERK2 itself, but have been seen three times in HLi1, twice in MK1 and twice in MK2. If such a mechanism operated, it would be expected that the tetraploid cells resulting would have just twice the number of chromosomes as the diploid stock from which they originated, and this appears to be the case at the 20th passage of ERK2, though by the 31st passage the polyploid cells had already slid back into the hypotetraploid region uniformly occupied by the older cell lines.

It is fair to conclude that though apparently full adaptation may occur in the diploid state, stability is not attained until the final chromosome pattern is

established in the hypotetraploid state. There is room for much further investigation as to why this should be so.

DISCUSSION

From the evidence presented it is possible to suggest the mechanisms of adaptation of cells to tissue culture. There is now overwhelming evidence for the belief that cellular development, including differentiation, depends on a complex series of interactions between the nuclear genes and the cytoplasm. These are thought to be mediated chemically by gene-products, possibly nucleic acids, which enter the cytoplasm from the nucleus to react with cytoplasmic constituents whose nature is determined by the past history of the cell and by its present environment. Products of the interaction re-enter the nucleus to react with the genetic material and modify its activity. While there is evidence, in such phenomena as evocation, that both the initiation and maintenance of differentiation depend on cytoplasmic influences, there is also evidence that the entire gene complex remains potentially active no matter how strong the suppression of particular genes in highly differentiated tissues. A full discussion of the evidence on which these concepts are based is given by Waddington (1956).

The overwhelming majority of cells in any normal mammalian tissue is diploid and presumably therefore of uniform genetic constitution, consequently the maintenance of those cytoplasmic differences which determine their state of differentiation must ultimately depend on the highly complex external environment to which each individual cell is subject. In culture all the normal *in vivo* relationships are disrupted and all cells subjected to a new and completely uniform macroenvironment. While local differences in cellular activity will produce local modifications of the environment for a time, cytoplasmic differences will be progressively eliminated during serial subcultivation and the overall effect will inevitably be towards greater uniformity of cytoplasmic constitution and hence of genetic activity. De-differentiation is uniformly observed during serial cultivation of tissue cells but it is not always appreciated that such de-differentiation is not towards a primitive cell type in the embryological sense, but to one whose ultimate nature depends on the artificial conditions of culture and therefore is unlikely ever to be encountered *in vivo*. On purely morphological grounds the ERK2 line would appear to have approached this state as early as the 6th passage, but its behaviour on dilution suggests that the process was by no means complete at this stage.

Progress towards uniformity could clearly be interrupted by any one of a number of theoretical mechanisms such as the presence of autonomous cytoplasmic determinants independent of nuclear control, but one mechanism which undoubtedly does modify such progress is the introduction of genotypic diversity as shown by the aberrations in chromosome numbers in the cell lines MK1 and ERK2.

From these basic considerations and our own experimental observations we would suggest that the early cultivation of cell lines derived from normal mammalian tissues may follow one of two paths. In parallel with the process of de-differentiation, genotypic diversity is introduced, probably by the occurrence of mitotic aberrations. If these aberrations do not lead to serious losses in chromosomes, resulting in fatal interference with metabolic functions, in a large proportion of cells, adaptation to *in vitro* conditions may follow smoothly in the near-diploid

population and polyploidy will later supervene as a secondary event whose significance remains in doubt. Transformation will not then occur. This is the sequence of events followed by ERK2.

If, on the other hand, early mitotic aberrations lead to heavy chromosome losses in a large proportion of the cells, as in the case of MK1, then the original diploid stock will lose vigour and eventually die out. The survival of the line must then depend on the adaptive capacity of these few cells with chromosome numbers in the diploid region and above whose metabolic systems remain intact. There is as yet no clue as to whether the cause of the mitotic aberrations is a medium deficiency or the presence of unsuspected toxic substances, but it is likely to be a factor directly affecting the nucleus and possibly connected therefore with nucleic acid metabolism. The sequence of events indicates that at this stage some form of adaptation is necessary before cells can multiply freely and transformation appears to be the mechanism by which this adaptation usually occurs, though the behaviour of MK1 indicates that it is not the only possible one.

The abrupt nature of transformation, together with the dramatic changes in morphology and behaviour which accompany the process suggest an underlying genetic mechanism, and the stability of the differences between MK1 and MK2 support this suggestion. Yet the reversibility of the change in MK3 indicates, for the reasons discussed on page 590, that the mechanism is not mutation. Further, the characteristic timing of the event indicates that it is linked to metabolic

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EXPLANATION OF PLATES.

FIG. 2.—Primary culture of monkey kidney.

FIG. 3.—MK1 line of untransformed monkey kidney cells. Confluent culture showing pattern of growth resembling primary culture.

FIG. 4.—MK1 line. Culture at earlier stage of development showing mutually oriented cells forming a lattice.

FIG. 5.—MK2 line of transformed monkey kidney cells showing typical unoriented growth.

FIG. 6.—MK1 line. Same culture as Fig. 3 at higher magnification to show cellular morphology.

FIG. 7.—MK2 line showing typical morphology of transformed cells.

FIG. 8.—MK3 line of transformed monkey kidney cells at 4th passage. Typical transformed morphology.

FIG. 9.—MK3 line at 26th passage following reversion. Cells now closely resemble those of the untransformed MK1 line shown in Fig. 6.

FIG. 10.—ERK2 line. Uniform spindle-cell sheet following inoculation at high density of 400,000 cells/flask or above.

FIG. 11.—ERK2 line. Differentiation into "epithelial" plaques and "fibroblastic" cells following inoculation at medium density of 200,000 cells/flask.

FIG. 12.—ERK2 line. First appearance of compact cells in culture seeded at low density 100,000 cells/flask.

FIG. 13.—ERK2 line. Same area as that shown in Fig. 12, 72 hours later showing early multiplication of compact cells to form plaque.

FIG. 14.—MK3 line at 26th passage. Low power view following reversion showing similarity of growth pattern to that of MK1 and primary culture (Figs. 2 and 3) and contrast with Fig. 5.

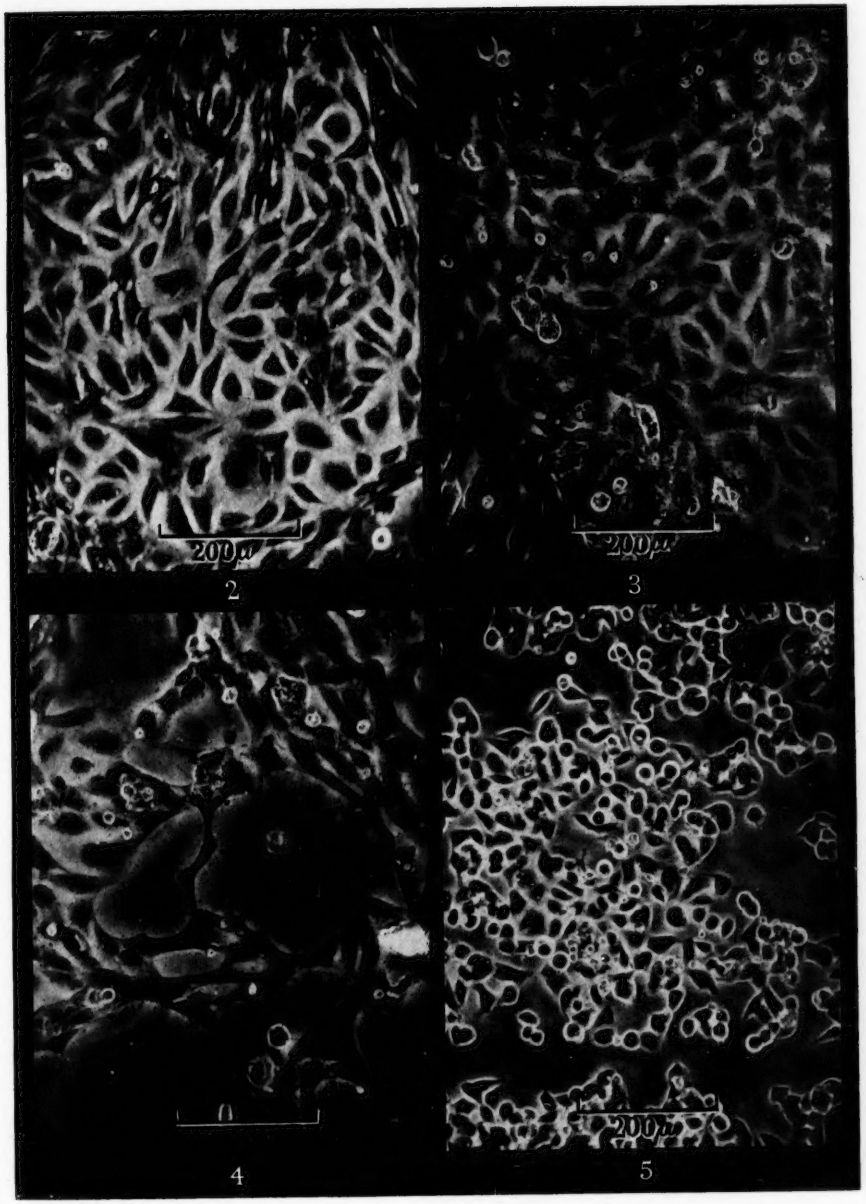
FIG. 15.—Untransformed ERK2 line. "Epithelial" cells showing typical granulation of cytoplasm.

FIG. 16.—Chromosome preparation from cell of MK1 line showing one minute chromosome (arrowed). Seventy-nine chromosomes.

FIG. 17.—Chromosome preparation from cell of human liver line, H.Li1. Endoreduplication. Seventy-five groups of chromosomes.

All photographs, except Figs. 16, 17, taken by phase-constrast illumination using Leitz Heine Condensor and Leitz optical system.

FIG. 16–17.—Preparations stained with aceto-carmine.



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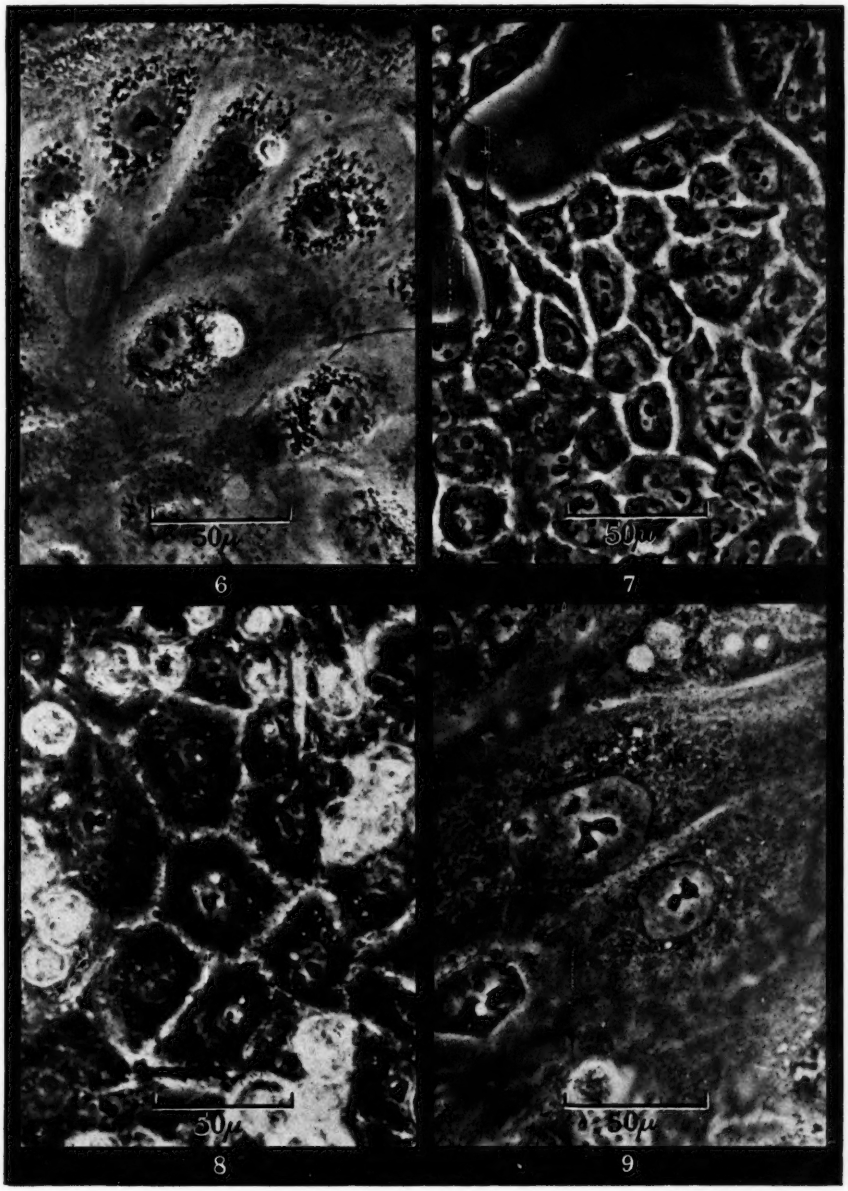
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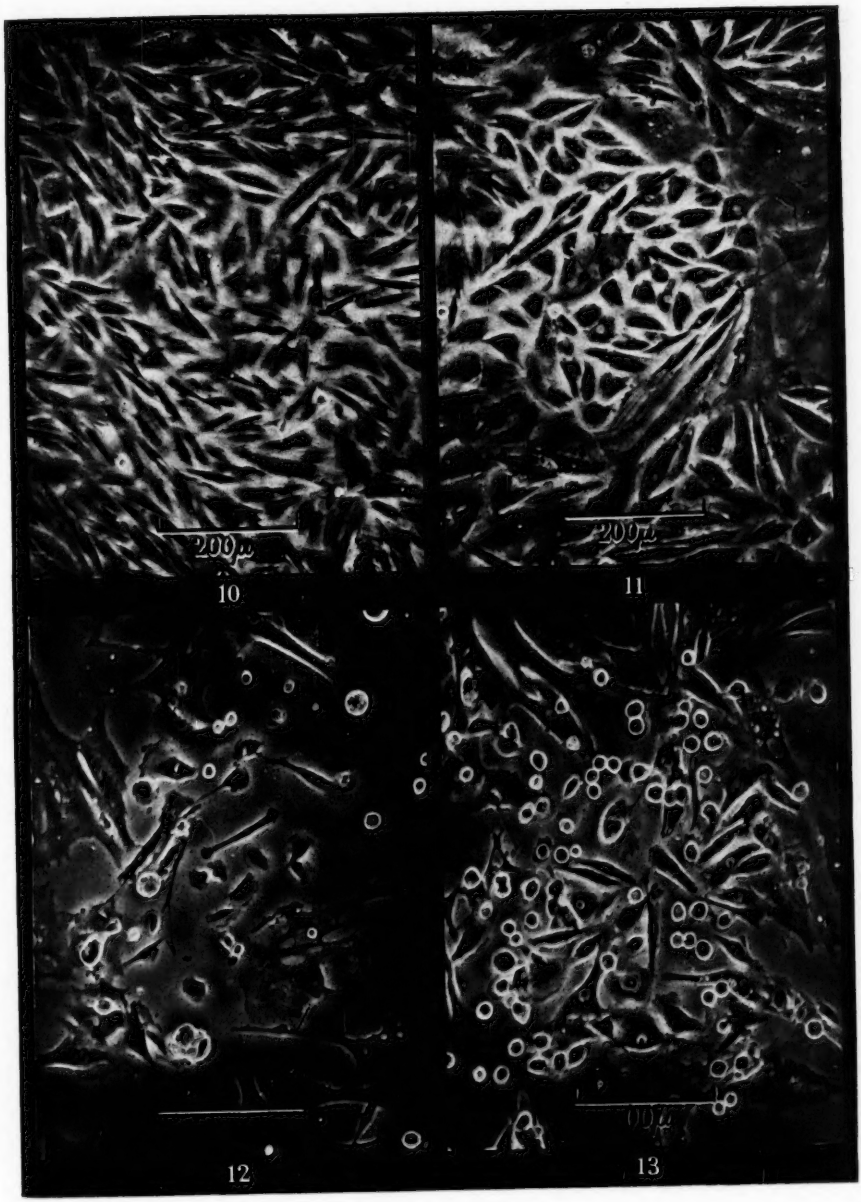
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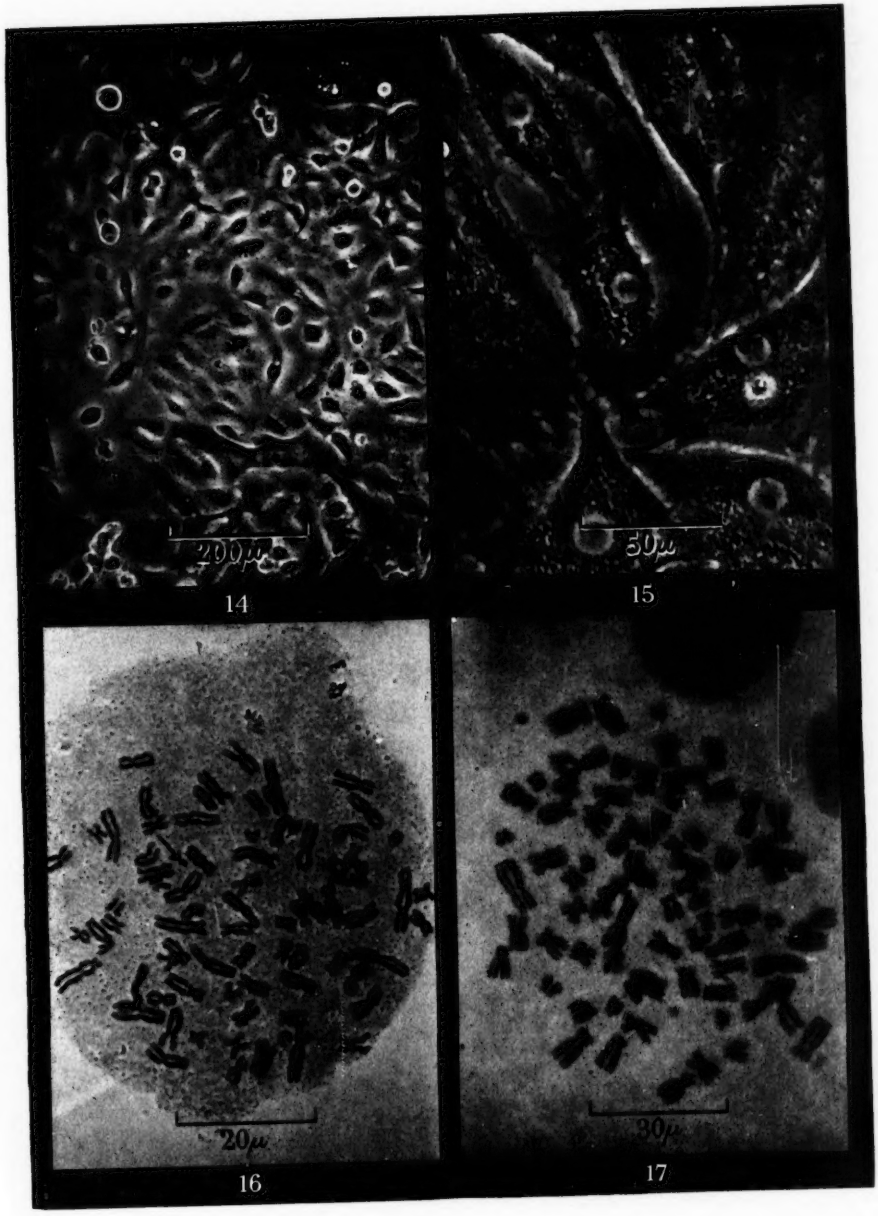

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failure of the cells and is therefore an impressed variation precipitated by the internal environmental state brought about by that failure. These various phenomena would be explained if the genetic mechanism in question consisted in a switch in the control of basic metabolism from one gene locus to another without alteration of the genotype.

Precedence for such a mechanism, involving transfer of control of a phenotypic character from one gene locus to another, has been established by Sonneborn (1954) and by Beale (1956) who demonstrated that control of the nature of the ciliary antigens of *Paramaecium aurelia* is vested in at least three gene loci of which only one is normally active at any one time. The activation of the appropriate locus is determined by the cytoplasmic state of the organism which may be altered by manipulation of the environmental conditions, control thus being switched from one locus to another at the will of the experimenter. A very similar mechanism has been postulated for the control of phase variation in *Salmonella* by Lederberg and Iino (1956). In this instance two loci are involved, H1, and H2 H2 being dominant in the sense that phenotypic expression of H1 depends on the state of activity of H2.

There is close similarity between these two systems and that which must be postulated to explain the phenomena of transformation. The known facts would be explained if a switch in metabolic control were precipitated, in genotypically competent cells only, by the critical cytoplasmic state brought about by metabolic failure. If the suppression of the orginal, or " O " locus, were complete or if this locus were subsequently lost by mitotic aberration, then the transformed, or " T " state would be stable, but if suppression were incomplete then reversion would be possible provided that O was dominant over T, as H2 is dominant over H1 in *Salmonella*.

Genetic mechanisms of the type here postulated can be adequately tested only if sexual or parasexual mechanisms can be utilised, or if techniques of transduction can be applied. Neither approach is as yet open for the investigation of cells in culture and stable genetic markers are as yet completely lacking. Critical investigation of the validity of this approach to the problem of transformation may, therefore, present great difficulty at first, but much that is at present puzzling in connection with the virus sensitivities of both transformed and untransformed cells could be explained on this basis, and metabolic studies on cells at various stages of cultivation might determine whether changes in metabolism in fact follow the pattern suggested by this hypothesis.

SUMMARY

Investigations have been carried out on the mechanism of cell " transformation " in tissue culture. There is a stable difference between the untransformed cell line MK1 and the transformed line MK2, which both originated from the same tissue culture stem. Reversion may take place from the transformed state to the untransformed. Transformation may be simulated by modification of the environmental conditions of the cells of an untransformed line. The pseudo-transformation so induced is not stable but reverts immediately when conditions are returned to normal. No antagonism exists between diploid cells in primary culture and transformed or untransformed cells. The chromosome numbers of all cell lines examined were found to lie in the hypo-tetraploid region, whether

or not the cells had undergone transformation. The acquisition of polyploidy was not related to the adaptation of the cells to *in vitro* conditions of growth, but followed as a secondary process.

These various findings cannot be explained on any simple hypothesis of mutation, adaptation, or the emergence of a cell type already present in primary culture. They are capable of explanation, however, on genetic grounds when considered in the light of what is known of the mechanisms of gene action. A hypothesis is therefore advanced to explain the known phenomena of transformation on the basis of this knowledge.

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OBSERVATIONS ON THE INCREASE IN SERUM BACTERICIDAL ACTIVITY IN THE PRESENCE OF CLINICAL DEXTRAN

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THE bactericidal activity of fresh normal serum against Gram-negative bacteria has been the subject of many investigations but the exact mechanism still remains unknown. A simple method of estimating bactericidal activity is to determine the highest dilution of serum that kills a given proportion of a standard inoculum of bacteria in a chosen time. Physiological saline or various buffers have been the diluents usually employed in these titrations. Recently, while comparing the suitability of various diluents, it was found that human serum in the presence of clinical dextran exerted bactericidal activity at much higher dilutions than when diluted in saline (Wardlaw, 1957). This *potentiation* of bactericidal titre by clinical dextran was contrary to expectation, since *native* dextran inhibits serum bactericidal activity. An investigation of the potentiating effect was therefore made and is reported here.

MATERIALS AND METHODS

Clinical dextrans.—Three different preparations were used :

Dextran M1, average mol. wt. 80,000 : from Cutter Laboratories, Oakland, California, U.S.A.

Intradex, average mol. wt. 170,000 : from Glaxo Laboratories, Greenford, Middlesex.

Dextraven, average mol. wt. 230,000 : from Benger Laboratories, Holmes Chapel, Cheshire.

Each was obtained as a sterile 6 per cent solution in normal saline, as used for transfusion.

Native dextran, with molecular weight in the region of 10^7 was obtained from The Commercial Solvents Corporation, Terre Haute, Indiana, U.S.A.

Bovine serum albumin was obtained as a sterile 30 per cent solution from Armour Laboratories, Hampden Park, Eastbourne, Sussex.

Normal serum.—Blood from healthy human donors and from normal rats and guinea-pigs was held for 1 hr. at room temperature and 18 hr. at 2°, after which the serum was separated and stored at −70°.

Properdin-deficient serum (*RPb*) was prepared from human serum as previously described (Wardlaw and Pillemer, 1956).

Bacteria.—Two strains were used. One, a strain of *Sh. shigae* ATCC No 9665, was obtained as strain No 377 from Dr. R. F. Parker, Department of Bacteriology, Western Reserve University, Cleveland, Ohio. This strain was sensitive to the properdin system in normal human serum (Wardlaw and Pillemer, 1956). The other was *Bact. coli* No. 2380, belonging to serotype $O_{111}B_4$, obtained from Dr. D. Rowley of this Institute. The characteristics of this strain have been described elsewhere (Rowley, 1954). Suspensions of organisms for bactericidal tests were made in normal saline either by emulsifying the growth of an 18-hr. nutrient agar slope culture, or by diluting a 5-hr. nutrient broth culture. Suspensions in each case were adjusted to contain approximately 25,000 viable cells per ml. and were added to the test mixtures of serum, etc. within a few minutes of preparation.

Titration of serum bactericidal activity

Serum dilutions of total volume 1·0 ml. were made by adding the required volume of undiluted or 1 : 10 serum to sterile tubes containing diluent. Bacteria (0·02 ml.) in normal saline were then added with a 0·2 ml. graduated pipette to these tubes and also to a tube containing 1 ml. broth, as non-bactericidal control. Viable counts were made immediately on the broth control and after 30 and 90 min. at 37° on the remainder, by spreading 0·1 ml. samples, taken with 0·2 ml. graduated pipettes, over the surfaces of nutrient agar plates. Plates were incubated overnight at 37° and colonies then counted. The bactericidal titre was taken as the dilution of serum killing 50 per cent of the inoculum, and was determined by interpolation on the graph of viable count against serum dilution.

RESULTS

The Potentiation of Serum Bactericidal Activity by Clinical Dextran

Potentiation of the bactericidal activity of a normal human serum by clinical dextran is illustrated in Fig. 1. Here three different clinical dextrans, of average mol. wt. 80,000, 170,000 and 230,000 were used, each at a concentration of 6 per cent in normal saline. *Sh. shigae* was the test organism. It is apparent that

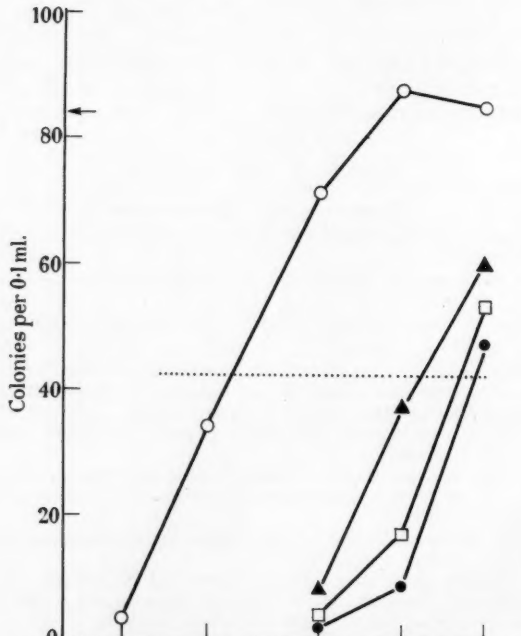

FIG. 1.—Bactericidal activity of normal human serum diluted in saline and in three different clinical dextrans. The test organism was *Sh. shigae* and the incubation time 90 min. ○ saline; ▲ "Intradex"; □ Dextran "M1"; ● "Dextraven". The arrow indicates the initial viable count, and the horizontal dotted line the 50 per cent survival level.

whereas with normal saline alone as diluent the titre of the serum was 1 : 12, with the dextrans " Intradex ", " M1 " and " Dextraven " it was respectively 1 : 50, 1 : 70 and 1 : 80. Samples of human serum from different individuals examined on different occasions all showed a 4- to 12-fold increase in bactericidal titre in the presence of clinical dextran. When normal rat and guinea-pig sera were substituted for human sera in these experiments, similar increases in bactericidal activity in the presence of dextran were observed.

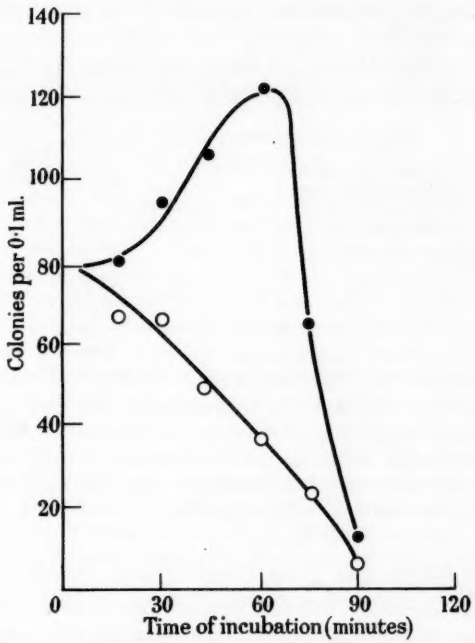

FIG. 2.—Death-time curves for *Bact. coli* suspended in 1/2 dilutions of serum in saline (●) and in clinical dextran (○).

Clinical dextran, in addition to extending the range of dilutions over which human serum was active in killing bacteria also increased the actual rate of killing by a 1 : 2 dilution of serum. Fig. 2 illustrates such an experiment in which *Bact. coli* was the test organism. These data show that the bactericidal action of serum in dextran was apparent at 30–60 min. whereas with serum in saline a slight *increase* in viable count occurred during this time. After 90 min., however, the two mixtures had almost equally low counts. The increase in count during the first 30–60 min. has also been observed with 1 : 5 and 1 : 10 dilutions of serum in saline, whereas the same dilutions in dextran showed only a progressive killing. Control tests showed that neither normal saline nor clinical dextran killed bacteria in the absence of serum during the period of the experiment.

Effect of dextran concentration

Having found that the undiluted (6 per cent) clinical dextran solutions substantially increased the bactericidal titre of serum, an examination was made of the effectiveness of lower concentrations. This was done by determining the 50 per cent killing dilutions of a human serum serially diluted in normal saline containing various concentrations of clinical dextran. The results in Table I show that the potentiating effect was rapidly lost as the dextran concentration was reduced. Thus 2 per cent dextran in saline was only one-fourth as effective as 6 per cent, and 0·5 per cent dextran was not distinguishable from saline alone.

TABLE I.—*Bactericidal Titre of Normal Human Serum Using Different Concentrations of Clinical Dextran as Diluent*

The test organism was *Sh. shigae*.

Concentration of clinical dextran in saline diluent (per cent)	Serum bactericidal titre
0	1 : 8
0·5	1 : 9
1	1 : 12
2	1 : 17
6	1 : 71

Comparison of clinical dextran with glucose, albumin and native dextran

In order to determine whether the potentiating effect of clinical dextran on serum bactericidal activity was related either to glucose content or to molecular weight, titrations of human serum bactericidal activity were made in 6 per cent solutions of glucose (mol. wt. 180), native dextran (mol. wt. approx. 10^7) and bovine serum albumin (mol. wt. 69,000). The results of these tests given in Table II

TABLE II.—*Serum Bactericidal Titres Using Clinical Dextran, Native Dextran, Glucose and Bovine Serum Albumin as Diluents*

The test organism was *Sh. shigae*.

Diluting medium	Serum bactericidal titre
Saline alone	1 : 12
Saline containing 6 per cent glucose	1 : 12
" " 6 " " clinical dextran	1 : 200
" " 6 " " native dextran	<1 : 2
" " 6 " " bovine serum albumin	1 : 24

suggest that both the molecular weight and the polysaccharide constitution of the clinical dextran are determinant factors. Native dextran, which has a much higher molecular weight, far from potentiating serum bactericidal activity, had the opposite effect of being strongly inhibitory. This result was also to be expected from the known anticomplementary properties of native dextran (Smith, Gallup and Stanley, 1952 ; Pillemer, Schoenberg, Blum and Wurz, 1955). Native levan was found to give a similar result (Wardlaw, unpublished observation). Glucose, on the other hand, which is the product of hydrolysis of native dextran neither

potentiated nor inhibited serum bactericidal activity. Bovine serum albumin gave only a slight potentiation compared with normal saline. This last finding suggested that a molecular weight of the same order as that of clinical dextran was not necessarily associated with ability to potentiate serum bactericidal activity.

Mode of Action of Clinical Dextran

In attempting to find out the mechanism by which clinical dextran potentiates serum bactericidal activity, the following possible modes of action were considered : (*a*) promotion of agglutination of the organisms, (*b*) potentiation of the activity of complement, (*c*) potentiation of the activity of properdin, and (*d*) direct action of clinical dextran on the bacteria, making them more sensitive to the bactericidal action of serum.

The promotion of agglutination by clinical dextran

Dextrans have been reported to promote certain agglutination reactions. Native dextran added to heparinized blood promotes rouleaux formation and rapid sedimentation of erythrocytes. Clinical dextran promotes agglutination of erythrocytes sensitized with incomplete Rh antibody (Jones, 1950). This reaction requires a critical dextran concentration close to 2 per cent and works best with dextran of mol. wt. 70,000. It was considered necessary in the present studies to examine the possibility that the reduction in viable count observed with serum diluted in clinical dextran might be due to agglutination of the organisms and not killing. Calculation shows that such agglutination would be an extremely improbable event since each cell in a suspension containing 10^3 organisms per ml. is separated from its nearest neighbour by approximately 1000 cell diameters. In order to observe possible agglutination it was therefore necessary to use a higher concentration of bacteria in the tests—an alteration which would also increase the probability of agglutination. Titrations of human serum in normal saline and in clinical dextran were therefore made using 10^6 bacteria per ml. After 30 and 90 min., samples were taken for viable counting and also for microscopic examination for agglutination. The viable counts showed that the clinical dextran had potentiated the bactericidal titre 6-fold over normal saline. Microscopically, however, no evidence of agglutination was observed even in preparations that subsequently were found to have undergone a 95 per cent reduction in viable count.

Another experiment bearing on the question of agglutination was made in which bacteria were exposed to sera which had been made non-bactericidal but which would be expected to contain agglutinins. The sera used were (*a*) normal human serum that had been de-complemented by treatment with hydrazine (Ecker, Pillemer and Seifter, 1943), or by heating for 30 min. at 56°, and (*b*) rabbit diagnostic antiserum to *Bact. coli* serotype $O_{111}B_4$ to which the strain 2380 belonged. The sera were diluted 1 : 20 in clinical dextran and in saline and their bactericidal activities determined. The results in Table III show that all the de-complemented sera not only failed to kill the bacteria but even permitted growth to occur. Furthermore, the increase in viable count occurred at approximately the same rate in saline as in clinical dextran, suggesting that agglutination was not taking place even between cells in close juxtaposition as a result of cell division.

TABLE III.—*Changes in Viable Count of* Bact. coli *Incubated with Non-bactericidal Sera Diluted in Saline and in Clinical Dextran*

Serum	Diluent	Viable count at 0 (colonies per 0·1 ml.)	60 min.	90 min.	150 min.
56° 30 min. human serum	Saline	18	20	54	126
	Dextran	25	21	60	140
N_2H_4-treated human serum	Saline	97	72	134	c.g.*
	Dextran	74	79	145	c.g.*
Rabbit Anti-seum $O_{111}B_4$	Saline	78	77	86	198
	Dextran	78	68	105	240

* c.g. = confluent growth.

The effect of dextran on haemolytic complement

Two possibilities were considered here : (*a*) that dextran might directly potentiate the activity of complement, and (*b*) that it might protect complement from the heat-inactivation that occurs when diluted serum is incubated at 37°. Haemolytic complement titrations on human serum were therefore made by a standard method (Ecker, Pillemer and Seifter, 1943) using, in parallel tests, normal saline

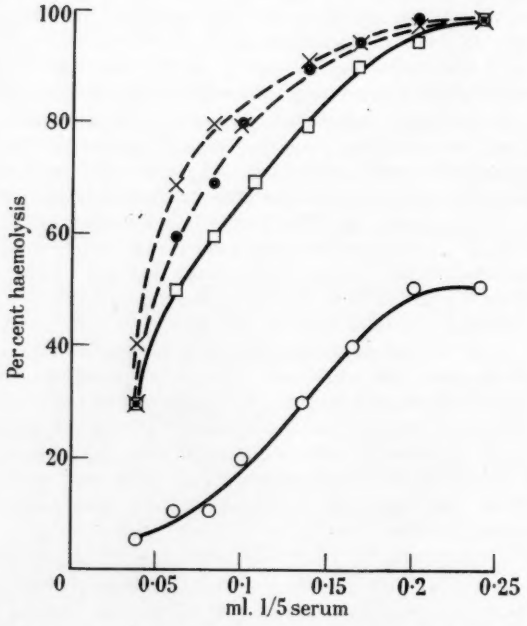

FIG. 3.—Haemolytic complement activity of normal human serum in saline and in clinical dextran.

– – × – – in saline.
——□—— in saline, 90 min. pre-incubation.
●– – –● in dextran.
——○—— in dextran, 90 min. pre-incubation.

and 6 per cent clinical dextran for diluting the serum and suspending the sensitized sheep erythrocytes. In the experiment to test whether dextran directly potentiated complement activity, the sensitized erythrocytes in either saline or dextran were added immediately to the serum dilutions in either saline or dextran respectively and the mixtures incubated 30 min. at 37°. The graph of per cent haemolysis against serum dilution is given in Fig. 3 and shows that the two diluents gave the same result. To detect the possible protective effect of clinical dextran on the heat-inactivation of complement, the dilutions of serum were incubated 90

FIG. 4.—Bactericidal activity of three samples of properdin-deficient serum diluted in clinical dextran. The test organism was *Sh. shigae* and the incubation time 90 min.

min. at 37° before addition of erythrocytes, after which a further 30 min. incubation was given. The results, also in Fig. 3, show that complement activity was lost more rapidly by incubation in dextran than in saline, indicating that dextran, far from preventing the heat-inactivation of complement at 37°, had, in fact, the opposite effect.

Effect of clinical dextran on the bactericidal activity of properdin

It was previously shown (Wardlaw and Pillemer, 1956) that the bactericidal activity of normal human serum on *Sh. shigae* No. 377 was due to the joint action of properdin, complement and Mg^{++}. Since complement was apparently not potentiated by clinical dextran and yet the overall bactericidal activity of serum was increased, it was considered possible that properdin might be the factor

affected. Data which could be interpreted as supporting this view were obtained while examining the effect of clinical dextran on serum which had been rendered properdin-deficient by zymosan treatment. These sera (RPb) were non-bactericidal when tested undiluted, or diluted in saline, but, because they contained complement, they become highly bactericidal when purified properdin was added. These properdin-deficient sera were also found to acquire bactericidal activity when diluted 1 : 2, 1 : 4 or 1 : 8 in clinical dextran (Fig. 4), in spite of the fact that the clinical dextran itself contained no properdin.

Effect of pretreatment of bacteria with dextran on their sensitivity to serum

The following experiment was made to determine whether pre-exposure of cells to clinical dextran caused any alteration in their sensitivity to serum bactericidal activity. A 5-hr. broth culture of *Sh. shigae* was diluted by a factor of 5×10^5 in clinical dextran and in normal saline, each containing 5 per cent of heat-inactivated serum. After 10 and 30 min. at 37°, 0·1 ml. of each diluted culture was rapidly transferred to 1 ml. dilutions of human serum in saline and, after a further 60 min. at 37°, viable counts made. The results in Table IV show that cells pre-exposed to clinical dextran were not more sensitive to serum than were cells pre-exposed to normal saline. This suggested that if any alteration in the sensitivity of the cells had occurred, it disappeared as soon as the cells were removed from the 6 per cent dextran medium.

TABLE IV.—*Effect of Pre-exposure of* Sh. shigae *Cells to Saline and to Clinical Dextran on their Subsequent Sensitivity to the Bactericidal Action of Serum*

Pre-exposure of cells at 37°	Viable count† after 60 min. at 37° in serum diluted in saline (colonies per 0·1 ml.)		
	1 : 10	1 : 20	1 : 40
10 min. saline* . .	7	107	103
30 min. saline* . .	10	103	98
10 min. dextran* . .	7	80	86
30 min. dextran* . .	13	110	114

† Initial viable counts : 90–105.
* Containing 5 per cent heat-inactivated (56° — 30 min.) serum.

DISCUSSION

A considerable number of polysaccharide-containing materials are known to inhibit the bactericidal activity of serum on Gram-negative organisms. The inhibitory activity has variously been attributed to removal or destruction of antibody, complement or properdin. Among the materials known to be active are zymosan (Dozois, Seifter and Ecker, 1943), heparin (Lambert and Richley, 1952), mucin (Spero, Waisbren, Schantz and Victor, 1951 ; Lambert and Richley, 1952), native dextrans and levans (Wardlaw, unpublished observations) and the O-antigens of Gram-negative bacteria (Thibault, 1939 ; Cundiff and Morgan, 1941). Compared with these materials, certain other polysaccharides such as starch, dextrin and inulin are relatively inert except at very high concentrations (Wardlaw, unpublished observations). Clinical dextran, on the other hand,

apparently falls into neither of these categories for it has the property, hitherto undescribed for a polysaccharide, of potentiating the bactericidal activity of serum.

The exact structural feature of clinical dextran that is responsible for this property has not been determined. However, molecular weight and polysaccharide constitution appear to be among the determinant factors. Thus native dextran with a 100-fold higher molecular weight inhibited serum bactericidal activity, whereas glucose with a 1000-fold lower molecular weight was inert. Protein of molecular weight similar to that of clinical dextran was also inert. Studies are in progress to determine more exactly the range of molecular weight over which glucose polymers show ability to inhibit or potentiate bactericidal activity, and also whether any particular pattern of glycosidic linkages is necessary.

Dextrans, both native and clinical have been shown to promote certain agglutination reactions of erythrocytes, and this suggested that similar phenomena might occur with bacteria. If this were the case, then the reduction in viable count produced by serum in the presence of clinical dextran could be attributed to agglutination and not killing. However, in none of a variety of experiments could promotion of bacterial agglutination by clinical dextran be detected. It was therefore concluded that in these studies a true potentiation of bactericidal activity was taking place. To account for the potentiation of bactericidal activity by dextran two further possibilities were considered. Firstly, clinical dextran might have a direct effect on the bacterial cells causing an alteration in their sensitivity to serum. For example, with the strains used in this investigation, log. phase cells were much more sensitive to the bactericidal action of serum than were cells in the lag phase, which suggested that dextran might potentiate the action of serum either by shortening the lag phase or by some other direct action on the cells. However, no evidence supporting this could be obtained.

The second possibility was that dextran might potentiate the action of one of the serum factors involved in the bactericidal process. That this factor is not haemolytic complement is clearly shown by the identical results obtained with complement titrations in normal saline and in dextran. Furthermore, prolonged exposure of serum to clinical dextran caused marked inactivation of complement. Attention was therefore turned to the possibility that properdin action was being enhanced. In this connection it was observed that properdin-deficient sera, which were non-bactericidal when tested undiluted or when diluted in saline, acquired definite bactericidal activity when diluted 1 : 4 in clinical dextran. A possible, but purely speculative interpretation is that properdin-deficient serum contains residual properdin whose interaction with bacteria is prevented by an inhibitor. This inhibitor cannot itself take part in the bactericidal process, but by virtue of having an affinity for sites on the bacterial surface close to, or identical with, those that combine with properdin, it blocks bactericidal action. That specific antibody has such an inhibitory action has already been shown (Wardlaw and Pillemer, 1956) and confirmed by Wedgwood (personal communication).

SUMMARY

Dilutions of normal human, rat and guinea-pig serum in clinical dextran have a higher bactericidal activity towards *Bact. coli* and *Sh. shigae* than do corresponding dilutions made in saline. This potentiation of serum bactericidal titre was

well shown using dextrans of mol. wt. 70,000 to 230,000, and at a concentration of 6 per cent. Lower concentrations were much less effective.

The potentiating effect of clinical dextran depends both on molecular weight and on polysaccharide constitution. Thus the effect was not produced by native dextran of much higher molecular weight, or with glucose or with bovine serum albumin, a protein with molecular weight similar to that of clinical dextran.

The mode of action of clinical dextran has not been elucidated. However, the potentiation of serum bactericidal activity is not due to promotion of agglutination or to preservation or potentiation of complement. Attempts to show that clinical dextran directly causes an increase in the sensitivity of the organisms to serum were unsuccessful.

I wish to thank Dr. Rowley for help and advice with this investigation. I am indebted to the members of the general public who gave blood used for this work; also to Dr. J. D. James, North London Blood Transfusion Centre, and Dr. J. G. Howard and Sister C. Wingard of this Institute who obtained it for me. Thanks are also due to Mr. P. Harwood for technical assistance.

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THE EFFECT OF PREVIOUS INJECTIONS OF TUBERCULOPROTEIN ON THE DEVELOPMENT OF TUBERCULIN SENSITIVITY FOLLOWING B.C.G. VACCINATION IN GUINEA-PIGS

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INTRODUCTION

THERE is now considerable evidence that both circulating antibodies (Coons, Leduc and Connolly, 1953 ; Ehrich and Harris, 1942 ; Fagraeus, 1948 ; Fagraeus and Grabar, 1953 ; Harris, Harris and Farber, 1954 ; McMaster and Hudack, 1935 ; White, Coons and Connolly, 1955) and the specific host substances or " antibodies " of bacterial allergy (Chase, 1945 ; Metaxas and Metaxas-Buehler, 1954) are formed in lymphoid tissue. It may even be that the same kind of cells are responsible for both types of immune response, but it is not known whether this is so.

In the case of circulating antibodies, it is possible to interfere with the primary response to an antigen by injecting it for the first time together with another antigen which has been previously administered (Barr and Llewellyn-Jones, 1953, 1955). The antibody-forming mechanism is apparently unable to respond properly to the new antigen because it is working almost to full capacity producing the antibodies of the secondary response to the other, previously administered, antigen. If, as we postulate, circulating antibodies are formed by the same cells as are the antibodies of tuberculin type allergy, then it might be possible to demonstrate a similar sort of interference between the production of circulating antibodies and the development of delayed-type skin sensitivity. An experiment designed to test this possibility is described in this paper.

Guinea-pigs were injected with a preparation of unheated tuberculoprotein which is known to cause formation of circulating antibodies (Boyden, 1957*b*) These animals, together with untreated guinea-pigs were then injected with living tubercle bacilli (B.C.G.) and the development of allergy in the two groups was compared. It was found that, when skin tested 11 days after the B.C.G. injection, all the " normal " vaccinated animals gave posititive delayed-type reactions, whereas the animals which had previously been injected with tuberculoprotein gave mild early-type, or Arthus, reactions. At a later skin test delayed-type reactions were seen in both groups. The interpretation of this result will be discussed.

MATERIALS AND METHODS

Guinea-pigs.—Female white guinea-pigs, weighing 300–500 gm., were used. They were bred at Hvidesten, the farm of Statens Seruminstitut.

Tuberculoprotein.—Culture filtrates from the human virulent strain E9656 were Seitz-filtered twice and then concentrated by ultrafiltration and lyophilized (Boyden and Sorkin 1955).

P.P.D. (*RT* 22).—This was obtained from Mr. Magnusson of the Tuberculin Department, Statens Seruminstitut. It had been prepared by trichloracetic acid precipitation of filtrates of heated cultures of tubercle bacilli of the human type (Lind, 1947, 1948).

B.C.G. vaccine was kindly supplied by Dr. Knud Tolderlund of the B.C.G. Department, Statens Seruminstitut.

Bovine albumin.—This was supplied by Dr. A. Hansen of the Physical-Chemical Department, Statens Seruminstitut.

Skin tests.—0·1 ml. of the antigen solution was injected intradermally on the back. The thickness of the skin at the site was measured immediately before the injection (Boyden, 1957*a*). The reaction was read at 3 and 48 hours afterwards, when intensity of erythema was assessed, and the diameter of the reaction and the skin thickness were measured. On each occasion the skin readings were made in random order, and the reader was unaware of the number and group of each animal as he read the test.

Serology.—Sera were tested in the haemolytic modification of the Middlebrook-Dubos test (Middlebrook, 1950; Middlebrook and Dubos, 1948). Details of the technique were as previously described (Boyden, 1957*a*).

EXPERIMENTAL

The effect of Previous Injection of Tuberculoprotein on theDevelopment of tuberculin sensitivity after B.C.G. injection

The experiment was set up with 2 groups of 20 guinea-pigs (Groups 1 and 2) and one group of 10 (Group 3). The treatment of the different groups was as follows:—

1*st day.*—Group 1 received 1 mg. tuberculoprotein in 1 ml. saline subcutaneously.
7*th day.*—Group 1 injected as on 1st day.
10*th day.*—Group 1 injected as on 1st day.
38*th day.*—Groups 1 and 2 were injected with 0·1 ml. of B.C.G. suspension (1 mg. per ml.) intradermally.
48*th day.*—5 ml. blood taken by heart puncture from first 8 animals in each group.
49*th day.*—All animals skin tested with 0·1 mg. P.P.D.
71*st day.*—All animals skin tested with 0·01 mg. P.P.D.

The results of this experiment which are given in Table I, show a big difference between the skin reactions of Group 1 and those of Group 2. Group 1, which had received tuberculoprotein several weeks prior to the B.C.G. injection, reacted with typical early-type reactions, seen particularly in the measurements of thickening of the skin at 3 hr.; by 48 hr. the reaction had almost completely disappeared. In marked contrast, all the animals of Group 2 had good reactions at 48 hr., while at 3 hr. the reactions of this group had been negligible.

In the haemolytic modification of the Middlebrook-Dubos test, all eight sera from Group 1, taken the day before the first skin test, were positive in titres ranging from 1/10 to 1/320 (the antigen fraction used for sensitizing the red cells was unheated and contained both α- and β-haemosensitins, the former being a polysaccharide and the latter probably a protein (Boyden, Sorkin and Engbaek, 1958).

The eight sera from Group 2 were all negative. On the occasion of the second skin test given 33 days after the B.C.G. vaccination, most of the animals in Group 1 showed evidence of an early-type reaction as previously. However, they also had typical tuberculin reactions at 48 hr., which were, on the average, only slightly less intense than those of Group 2.

The possibility that the early-type reaction in Group 1 might have influenced the course of the delayed reaction by causing an immediate increase in capillary

permeability which allowed tuberculoprotein or toxic products of the reaction to escape from the site was tested in the next experiment.

TABLE I.—*Reaction to Skin Tests* 11 *Days after B.C.G. Vaccination and Antibody Titres in Experiment* 1

Number of guinea-pig	Group	Injection	3 hr.		48 hr.		Serum titres
3983	1	Tuberculo-	−	17	±6	10	40
3984		protein and	±18	18	±6	17	40
3985		B.C.G.	±20	19	±5	6	10
3986			±17	29	−	13	40
3987			−	21	±5	14	40
3988			±17	23	−	12	40
3989			±14	18	−	13	20
3990			±20	17	+4	12	320
3991			−	13	++9	14	—
3992			±22	30	±4	11	—
3993			−	10	+5	6	—
3994			±20	19	+7	9	—
3995			±21	29	+5	10	—
3996			+8	17	−	12	—
3997			−	21	++4	4	—
3998			−	8	++11	9	—
3999			−	15	+6	6	—
4000			±21	17	−	11	—
4001			±18	12	++6	13	—
4002			−	10	±6	7	—
Average	..	..	..	18·2	..	10·5	68·8
4003	2	B.C.G.	±15	13	+++13	18	0
4004			±6	9	+++12	18	0
4005			−	11	++++22	15	0
4006			−	10	++++15	22	0
4007			..	..	..	..	0
4008			−	9	+++15	19	0
4009			−	5	++++13	16	0
4010			−	11	+++14	17	0
4011			−	7	+++14	15	—
4012			−	13	+++12	11	—
4013			±5	9	++12	12	—
4014			±7	7	++++16	19	—
4015			−	9	+++16	16	—
4017			−	10	++++15	21	—
4018			±5	9	+++13	15	—
4019			−	10	++++18	20	—
4020			±15	12	++++14	21	—
4021			−	8	++++18	20	—
4022			−	10	++++19	12	—
Average	..	..	..	9·6	..	17·1	0

Key : ± = a trace of erythema.
\+ = pale pink.
++++ = intense erythema.

Note on skin readings.—The colouration of the skin reactions are denoted by signs as explained in the key. In each instance the number which follows the sign indicates the diameter in millimetres of the coloured zone. The number given in the separate column indicates the difference in millimetres between the thickness of the skin at the time of reading and the thickness immediately before the injection of P.P.D. There were no skin reactions in the control (non-vaccinated) group (Group 3).

Serum titres.—The figures refer to the reciprocal of the highest dilution giving complete haemolysis. 0 means there was no complete haemolysis in any tube. — means not tested.

The Effect of an Early-type Reaction on a Delayed-type Reaction at the Same Site

In the second experiment three groups were treated as in previous experiment. A fourth group received injections of bovine albumin instead of tuberculoprotein 4–5 weeks before the B.C.G. injection.

A skin test was performed as previously, 11 days after the injection of B.C.G. On this occasion all animals were skin tested at 3 sites, with (*a*) P.P.D., (*b*) bovine albumin, (*c*) a mixture of P.P.D. and bovine albumin.

1*st day*.—Group 1 injected with 1 mg. tuberculoprotein in 1 ml. saline subcutaneously.
Group 2 injected with 4 mg. bovine albumin in 1 ml. saline subcutaneously.
7*th day*.—As on 1st day.
10*th day*.—As on 1st day.
40*th day*.—Groups 1, 2, and 3 injected with 0·1 ml. B.C.G. suspension (1 mg. per ml.) intradermally.
51*st day*.—All animals skin tested in 3 places with
(*a*) P.P.D. 1 mg. per ml.
(*b*) Bovine albumin 1 mg. per ml.
(*c*) A mixture containing P.P.D. 1 mg. per ml. and bovine albumin 1 mg. per ml.

The results of this test are given in Table II.

In the first place, these results confirm those of the first experiment, in that most of the guinea-pigs injected with tuberculoprotein 4–5 weeks before B.C.G. (Group 1) had negligible reactions at 48 hr., at which time most of the animals which had received only B.C.G. (Group 2) showed strong positive reactions. The animals in the former group had typical early-type reactions to P.P.D.

As was expected, the animals which had been previously injected with bovine albumin (Group 3) also gave typical early-type reactions to bovine albumin, whether it was injected alone or with P.P.D. (These early reactions are more evident in the readings of skin thickness than in the erythema readings.) The significant fact is that in this group the tuberculin reaction (at 48 hr.) was in all cases as strong, or almost as strong, when the tuberculin was injected with bovine albumin as when it was injected alone. Although the Arthus reactions to the bovine albumin were not quite as strong as those against the tuberculin (Group 1), this result strongly suggests that an early-type reaction does not seriously interfere with the development of a tuberculin reaction at the same site.

TABLE II.—*Skin Reactions in Experiment 2*

Number of	Reactions to P.P.D.		Reaction to bovine albumin		Reactions to mixture of P.P.D. and bovine albumin	
	3 hr.	48 hr.	3 hr.	48 hr.	3 hr.	48 hr.

DISCUSSION

The experiments described above show that guinea-pigs which received injections of tuberculoprotein some weeks prior to B.C.G. injection exhibited no delayed type sensitivity to tuberculin 11 days after vaccination. They did, however, react to P.P.D. with early-type reactions, due to sensitization by the previous injections of tuberculoprotein (Boyden, 1957*a*). In contrast, guinea-pigs which had not received previous injections of tuberculoprotein, gave strong delayed-type reactions to the same dose of P.P.D. 11 days after B.C.G. vaccination.

In trying to interpret this result, it was necessary to consider the possibility that the existence of an early-type reaction due to the sensitization by tuberculoprotein might influence the tuberculin reaction by allowing tuberculoprotein or products of cell damage to escape readily from the site. It has been shown by

TABLE II.—*Skin Reactions in Experiment 2*

Number of guinea-pig	Group	Injection	Reactions to P.P.D. 3 hr.		Reactions to P.P.D. 48 hr.		Reaction to bovine albumin 3 hr.		Reaction to bovine albumin 48 hr.		Reactions to mixture of P.P.D. and bovine albumin 3 hr.		Reactions to mixture of P.P.D. and bovine albumin 48 hr.	
6353	1	Tuberculo-protein and B.C.G.	±10	11	−	3	−	3	−	−1	±12	2	−	2
6354			±25	22	±7	8	−	5	−	1	±22	22	−	10
6355			−	9	−	12	−	2	−	0	−	16	−	3
6356			−	13	−	4	−	3	−	0	−	12	−	3
6357			+8 ±26	27	−	3	−	2	−	0	++6 ±30	28	−	5
6358			±14	14	±5	6	−	2	−	2	±15	17	±5	6
6359			±16	16	−	7	−	2	−	0	±20	18	−	9
6360			+6 ±21	24	−	6	−	3	−	−1	+4 ±25	24	−	11
6361			+21	18	−	7	−	2	−	0	+27	21	±5	8
6362			±16	14	±8	5	−	1	−	−1	±16	17	±10	7
Average	..	..	..	16·8	..	6·1	..	2·5	..	0	..	17·7	..	6·4
6363	2	Bovine albumin and B.C.G.	+3 ±10	6	+++12	11	±12	11	−	−1	−	17	+++15	13
6364			−	9	++12	13	±10	15	−	0	±10	16	+12	9
6365			−	7	+14	9	−	18	−	1	−	16	+11	11
6366			±10	9	+++14	11	±10	16	−	2	±12	18	+++14	17
6367			−	8	+10	7	−	7	−	1	−	13	+10	11
6368			−	8	±3	3	±16	13	−	−1	±16	15	±6	4
6369			−	5	++11	4	−	17	−	−2	−	15	+11	5
6370			−	7	++11	8	−	14	−	0	−	13	+11	6
6371			−	5	++14	6	±13	18	−	0	±16	16	+10	8
6372			−	8	++11	10	±16	14	−	1	+5 ±16	16	++11	14
Average	..	..	..	7·2	..	8·2	..	14·3	..	0·1	..	15·5	..	9·8
6373	3	B.C.G.	−	6	++10	10	−	6	−	1	±10	7	+10	7
6374			±14	9	++12	11	−	5	−	1	−	9	++12	8
6375			−	8	+9	10	−	6	−	2	±5	7	+7	6
6476			±11	5	++9	10	−	6	−	2	++7	12	++11	15
6377			−	7	+++12	8	−	4	−	1	±6	11	+++10	10
6378			±6	9	±4	4	−	4	−	−1	−	8	±4	5
6379			−	6	+6	6	−	4	−	−2	±10	11	+9	11
6380			−	7	++11	9	−	4	−	0	+3 ±10	14	++13	16
6381			−	7	++12	16	−	3	−	1	±5	12	++12	16
6382			±6	5	+8	6	±3	4	−	0	±6	11	++9	1
Average	..	..	..	6·9	..	9·0	..	4·6	..	0·5	..	10·2	..	9·5
6383	4	No treatment	−	5	−	4	−	5	−	4	±5	8	−	2
6384			−	6	−	0	−	6	−	−1	±5	8	−	1
6385			±	11	−	2	−	7	−	0	±7	9	−	0
6387			−	5	−	1	−	5	−	0	−	7	−	1
6388			±4	9	+	1	−	8	−	−1	±7	8	−	−1
Average	..	..	..	7·2	..	1·6	..	6·2	..	0·4	..	8·0	..	0·6

Key: ± = *a trace of erythema.* + = *pale pink.* ++++ = *intense erythema.*

Note on skin readings.—The colouration of the skin reactions are denoted by signs as explained in the key. In each instance the number which follows the sign indicates the diameter in millimetres of the coloured zone. The number given in the separate column indicates the difference in millimetres between the thickness of the skin at the time of reading and the thickness immediately before the injection of P.P.D.

Pepys (1953) that the intensity of tuberculin reactions can be greatly reduced by injecting histamine diphosphate into the site immediately after the injection of tuberculin. However, the second experiment described above seems to speak against this interpretation; for it was shown that when bovine albumin and P.P.D. were injected into the same site in animals sensitized to the former antigen, the early-type reactions to bovine albumin had little, if any, influence on the intensity of the subsequent delayed reaction to P.P.D.

There seem to be two other possible interpretations of the results, and it is difficult at the present state of our knowledge to choose between them. The first, which was in mind when the experiment was set up, is that the injection of B.C.G. into the animals which had previously received tuberculoprotein causes a secondary response of circulating antibodies against tuberculoproteins, thereby interfering with the ability of the antibody-producing mechanism to form "allergy antibodies" against these antigens.

The other possibility is that, although the "allergy antibody" is formed at the same rate in both groups of guinea-pigs, there is competition between circulating antibodies and "allergy antibodies" for antigen in the animals which had previously received tuberculoprotein. When the level of the allergy is relatively low, as it is 11 days after vaccination, circulating antibodies in high titre might well tend to depress the delayed allergic reaction by combining with and "neutralizing" the antigen.

The first interpretation is in accord with the hypothesis that circulating gamma globulin antibodies, and the "antibodies" of allergy are produced by the same mechanism and in the same cells. Possibly the factor which determines which kind of antibody will be formed is the particular form in which the antigen is received by the antibody-forming cells. If protein antigen is injected in solution, it is likely to reach these cells in an unmodified form. But in tuberculous infection, for example, the protein antigen must necessarily pass through macrophages before reaching the antibody-forming cells and may well be modified in some way by the process. The cells, on receiving this modified antigen, could be expected to respond to it differently (Boyden, 1957*b*).

The second interpretation, that the antibodies responsible for the Arthus type of reaction compete for the antigen with those responsible for tuberculin allergy, raises the question as to what extent such competition might play a part in tuberculous disease. Is it possible that in tuberculosis high titres of circulating antibodies which are thought to be ineffective against tubercle bacilli, might actually be prejudicial to the interests of the host by "protecting" the bacilli against the possibly more anti-bacillary effects of the allergy antibodies of the same specificity?

The various problems discussed above are being investigated in a further series of experiments.

SUMMARY

Guinea-pigs skin tested with tuberculin (P.P.D.) 11 days after B.C.G. vaccination gave strong delayed-type skin reactions. However, guinea-pigs which had received injections of tuberculoprotein 4–5 weeks before the injection of B.C.G. were not tuberculin positive 11 days after vaccination. They did, however, show mild skin reactions of the early type due to sensitization by tuberculoprotein.

An early-type skin reaction to bovine albumin at the same site as a tuberculin reaction had little or no influence on the latter reaction.

The interpretation of these results is discussed.

This work was carried out with the technical assistance of Miss Gabrielle Hurter and Miss Jytte Mensen.

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THE ESTIMATION OF BASAL BLOOD PRESSURE IN THE RAT BY TAIL OSCILLOSCOPE

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MANY methods have been described for measuring the blood pressure of rats by procedures which avoid direct arterial puncture. They all depend on detecting the swelling of a part or a pulse in one or other appendage. It seems probable that a number of these methods measure the prevailing blood pressure with reasonable accuracy. Determinations of " casual " blood pressure, however accurate, have not the value of accurate determinations made under readily reproducible basal conditions. When blood pressure is measured clinically, care is taken to see that the subject is physically and mentally relaxed, but even with elaborate precautions it is not easy to arrive at basal conditions (Pickering, 1955). The problem is no easier for the experimentalist. Small laboratory animals must be restrained in comfort while blood pressure measurements are taken. When the tail is used to measure blood pressure, the rat can assume a natural sitting posture throughout the period of restraint; hence methods using the tail have a great advantage in this respect. However, at room temperatures the circulation in the rat's tail is intermittent and the animal has to be warmed to promote an adequate tail circulation. Care has to be taken that this procedure does not itself cause any disturbance of blood pressure.

Of the procedures which use the tail, the plethysmographic method introduced by Byrom and Wilson (1938) and modified by Williams, Harrison and Grollman (1939) is probably the one most frequently chosen. Byrom (1947) later described a tail oscilloscope which has many advantages over the plethysmographic method. He originally recommended that blood pressure should be measured during the course of a light ether anaesthetic. Although Byrom has compared blood pressure determinations by the tail oscilloscope with recordings of mean arterial pressure from an intra-arterial cannula (personal communication), these results were not published.

The present communication establishes the accuracy of the tail oscilloscope as a measure of systolic blood pressure, and examines some of the factors thought to disturb basal conditions when determining blood pressure by the tail oscilloscope.

METHODS

The tail oscilloscope.—The principle of the tail oscilloscope is that of the clinical sphygmomanometer. The armband is replaced by an air-pressure cuff (" occluding cuff ") on the base of the tail and the stethoscope by another distally placed air cuff (" pick-up " cuff). Pulsations of the caudal artery are conducted from the " pick-up " cuff to a sensitive rubber

tambour carrying a tiny peripherally placed mirror, which deflects a beam of light. The oscillations of this optical lever are observed on a ground glass screen. The amplitude of the oscillation on the screen (" pulse amplitude " for the purpose of this paper) is a measure of the caudal pulse pressure The blood pressure is measured by raising the pressure in the proximal cuff until the pulsations transmitted from the distal cuff cease. The pressure in the occluding cuff is then allowed to drift down. The pressure in the occluding cuff when the oscillations of the light beam become continuous is recorded as the blood pressure

Rats.—Adult male and female albino rats bred in this laboratory from stock of Wistar origin were used.

Restraint cages.—These were made from cylindrical glass tubing about 2½ in. (6·25 cm.) in diameter. The front end was drawn down to a hole which would admit only the snout of the rat. Tubes of various diameters and lengths were made to accommodate rats of various sizes. The cage should be just narrow enough to prevent the rat from turning round. The open end of the cage was closed by a radially split rubber bung with a central hole through which the tail and thermocouple leads passed without constriction.

Control of body temperature.—The temperature of individual rats was controlled by observation of the rectal temperature. This could be measured to 0·02° C. by an indwelling thermocouple. The tip of the copper-constantin junction was embedded in a spherical mass of solder about 2 mm. in diameter. The last 2 in. (5 cm.) of the thermocouple were sheathed in plastic tubing (1 mm. internal diameter). The sheathed thermocouple was introduced at least 4 cm. into the lower intestine and held in place by splinting it to the base of the tail with a turn of adhesive tape. The stiffening provided by the plastic sheath prevented ejection of the thermocouple during defaecation. The temperature was measured by balancing the thermal e.m.f. against a dry cell, and adjusting the resistance of a potentiometer circuit so that a sensitive spot galvonometer gave a direct reading in degrees centigrade on the galvonometer scale.

Calibration of the tail oscilloscope.—At first it was assumed, as have most workers in the past when calibrating such apparatus, that the systolic pressures measured by the tail oscilloscope would be parallel to but at a slightly higher level than mean carotid pressure. Hence a comparison of tail readings and mean carotid pressure measured with a mercury manometer would be an adequate check on the accuracy of the machine. It was soon found to be inadequate. Comparisons were made in a series of rats in this way but it was impossible to sustain an adequate caudal circulation for any length of time in anaesthetised rats even though they were kept warm.

The following method overcame these difficulties. A polythene tube, 0·5 mm. in bore and 15 cm. long was inserted into the common carotid artery and the free end was brought out at the back of the neck and connected to an electromanometer. When the rat recovered from its anaesthetic, it usually sat quietly in the restraint cage. The pulse obtained in the tail was of the same amplitude as occurs in unoperated rats, and readings could be taken for as long as desired. Clotting in the cannula was prevented by the periodic injection of 0·05 ml. or less of heparin solution containing 250 units per ml. It was not found necessary to inject systemically effective doses of heparin and hence there was no bleeding from the neck. By using normal and renal hypertensive rats, and by giving injections of renin, it was possible to make blood pressure comparisons over the range of 80 to 250 mm. Hg. Renin was prepared by Schales' (1942) method. It was injected intravenously or intra-arterially. The dose was not accurately calculated but was sufficient to cause blood pressure rises often of 80–90 mm. Hg. Following the injections, a raised but slowly falling blood pressure was maintained for an hour or more.

The electromanometer consisted of a " Southern Instruments " capacitance type transducer and a frequency modulated pre-amplifier (Atree, 1955) which fed a directly coupled main amplifier driving a " Both " pen recorder. When mean pressures were required an electronic integrator was introduced between the pre-amplifier and main amplifier. During each experiment frequent calibrations of the manometer were carried out to avoid errors from thermal drift. Each time a reading of blood pressure was made by the tail oscilloscope a signal was injected into the electromanometer to indicate the instant at which the indirect reading was made on the tail.

Using unanaesthetised rats and the electromanometer further comparisons of tail readings with mean pressures and later with systolic pressures in the carotid artery were carried out. For the calibration of the tail oscilloscope a total of 14 rats were used, and 600 comparisons of direct and indirect readings were made.

RESULTS

Comparison of tail readings and mean carotid pressure

About 350 simultaneous comparisons of mean carotid pressure and tail readings have been plotted in Fig. 1. (For reasons to be discussed later, readings at pulse amplitudes of less than 3 mm. were excluded.) Although the direct and indirect

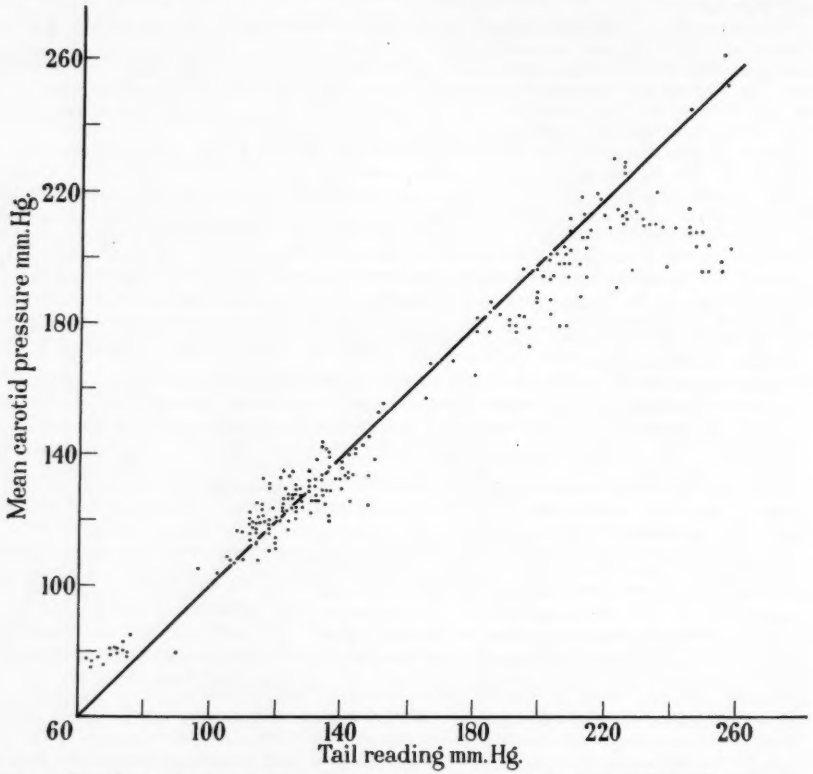

FIG. 1.—Correlation between intra-arterial measurements of *mean* carotid blood pressure and oscilloscope readings. Note that the readings from the tail overestimate the mean blood pressure in the upper range.

readings were fairly close in the range of normal blood pressures, at the top of the hypertensive range almost all the tail readings exceeded those of mean pressure. Most of the high readings were obtained after the injection of renin and were invariably associated with pulse oscillations of large amplitude.

Comparison of tail readings and systolic carotid pressure

When systolic carotid pressure and tail readings were compared it was found that such large pulse oscillations indicated a high pulse pressure (systolic-diastolic

pressure difference). Indeed the pulse amplitude estimated from the tail oscilloscope correlated quite well with the pulse pressure. Moreover it was now found that the direct reading and tail reading agreed very closely over the full blood pressure range in each of four animals (Fig. 2). Clearly the difference between the tail reading (estimating systolic pressure) and the mean pressure found

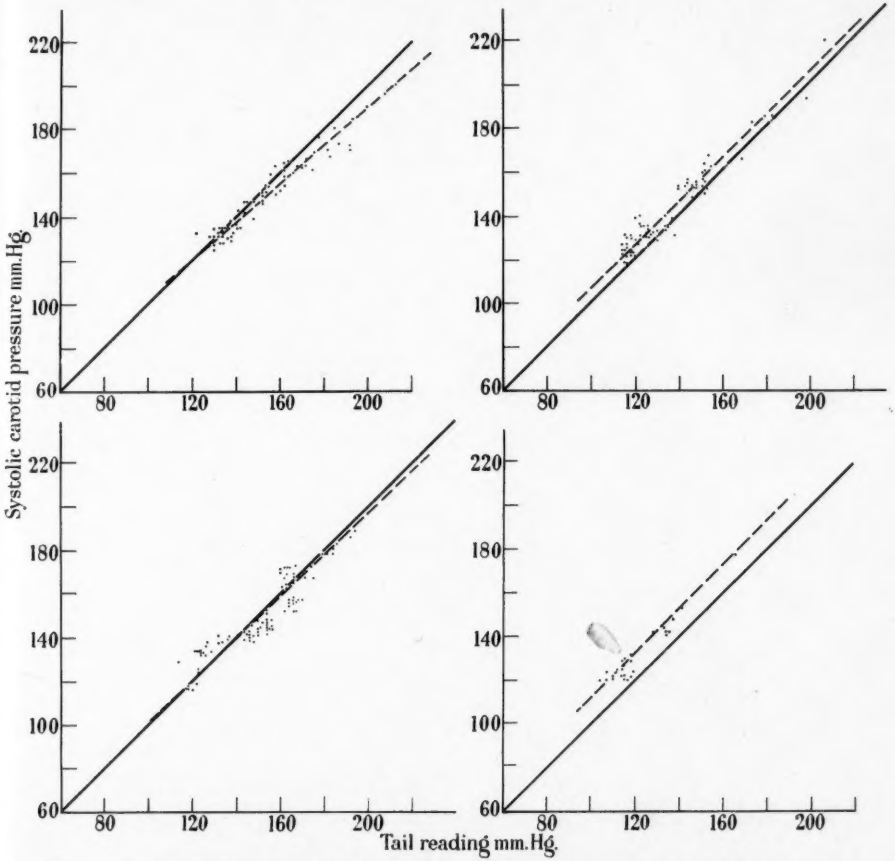

FIG. 2.—Correlation between *systolic* carotid blood pressure and readings by the tail oscilloscope. The dotted line is the calculated regression for the data.

previously, resulted from the greater difference between systolic pressure and mean pressure when the pulse pressure is large.

On the other hand when the amplitude of the pulse from the tail was small, the tail reading gave an underestimate of arterial pressure. This was due to constriction of the caudal artery. The pulse which first appeared when a rat was gently warmed was of small amplitude, but gradually it increased. Blood pressure

estimates during this period showed an apparent progressive rise (Fig. 3). A simultaneous record from the carotid artery demonstrated that the true systolic pressure was not measured by the tail oscilloscope until the tail pulse amplitude had increased to 3–4 mm. Clearly, estimates of blood pressure can be made on the tail only if there is sufficient dilatation of the caudal artery.

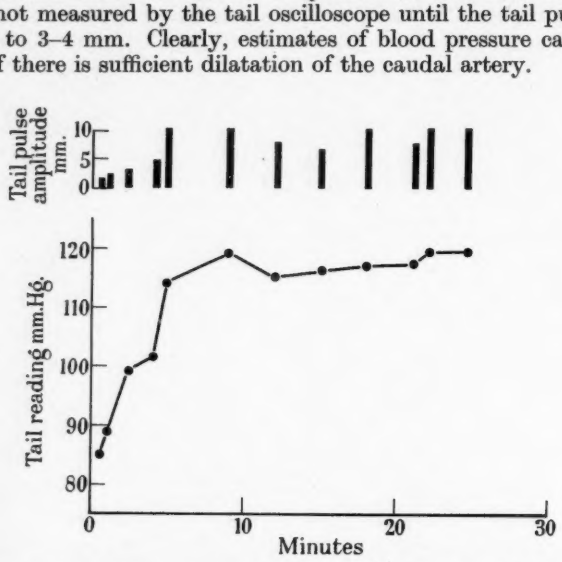

FIG. 3.—Blood pressure measurements recorded as a rat was warmed and the pulse appeared in the tail. Note that as the amplitude of the pulse increases, the blood pressure measurements show an apparent rise.

Influence of body temperature

Attempts to ensure an adequate caudal pulse by heating the tail alone were unsuccessful, unless in the process the body temperature also became elevated. When whole body heating was used to increase the tail circulation, the rate at which the body temperature was raised proved important. Rapid heating frequently induced restlessness and some elevation of blood pressure above basal levels. The time taken for a pulse to appear after the rat was placed in the restraint cage was less, if previously the animals had been housed in warm quarters (about 21–24°). The body temperature at which an adequate caudal pulse appeared was very variable between the limits of about 36·5°–39°. Immediately following surgery, there was little difficulty in obtaining an adequate tail pulse, except in animals which had been allowed to bleed, or in which body temperature had not been maintained.

Effects of over-heating

In confirmation of previous investigators (Proskauer, Neumann and Graef, 1945; Olmsted, Corcoran and Page, 1951), it was found that excessive heating caused a rise in blood pressure which was usually more marked in hypertensive animals. This is illustrated in Fig. 4 which also shows that a relatively wide range (about 2°) existed between the temperature at which a pulse first appeared and that at which a thermal pressor effect was seen. In the majority of animals

a temperature of 39° to 39·5° appeared to be the safe upper limit. It was easy, by periodically adjusting the rate of heat input, to maintain the body temperature of a rat within limits of ± 0·2°. Under these conditions the blood pressure remained surprisingly steady for periods of up to 8 hours.

The occurrence of Sigmund-Mayer waves

Periodic fluctuations in the level of blood pressure known as Sigmund-Mayer waves are, as stated by Byrom (1947), seen in some rats during blood pressure determinations. In normotensive rats these have almost always been an indication of excessive heating or excitement. Sigmund-Mayer waves were observed more

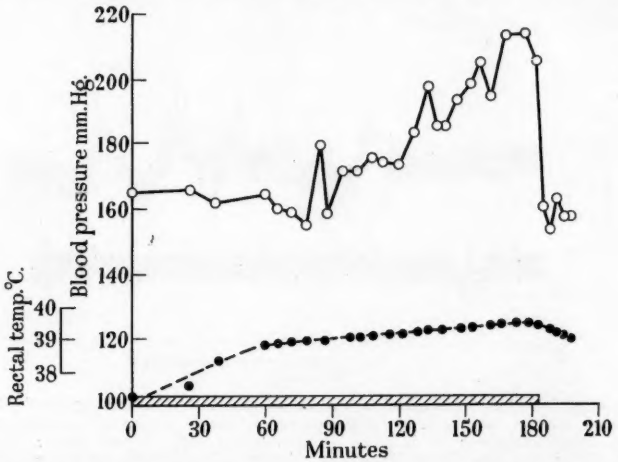

FIG. 4.—The pressor effect of overheating a hypertensive rat. The hatched area indicates duration of heating, (●——●) rectal temperature and (○——○) blood pressure measurements by tail oscilloscope.

frequently in hypertensive rats. Their presence is shown in the following way. As the pressure in the occluding cuff is lowered from above systolic pressure, the pulses at the peak of the Sigmund-Mayer waves pass in short intermittent volleys. As the pressure is further reduced the volleys merge to form a continuous pulse oscillation. Under these circumstances the level at which blood pressure is recorded is somewhat arbitrary. The most suitable point appears to be when the pulse becomes continuous. When hypertensive rats are under basal conditions the height of the Sigmund-Mayer waves is seldom more than 5–10 mm. When they are excited or overheated the waves may be 40 mm. from crest to trough.

The effect of training

Analysis of repeated blood pressure reading on the same group of animals showed that on the first occasion on which the blood pressure was measured, the mean basal blood pressure (calculated from 6 to 10 consecutive readings) was significantly higher than on subsequent occasions. This difference was of the order of 10 mm. Hg, but occurred in only about half the rats.

The effect of disturbing the animal

It has been stated that " even with trained rats meticulous care must be taken to avoid rough handling, injections and unusual voices and smells. Quite minor lapses often not apparent to the operator will excite a rat and raise its blood pressure by 40 mm. or more " (Byrom, 1947 ; quoting evidence from Kempf and Page, 1942). While these are good recommendations and should be observed whenever possible, it is felt that they are much less warranted than this statement would suggest. With the strain of Wistar albino rats used in this laboratory, no evidence has been found that unusual voices or noises significantly affected the level of blood pressure. Even rough handling or the injection of non-pressor substances produced only a minor and transient disturbance of blood pressure which was over in a few minutes.

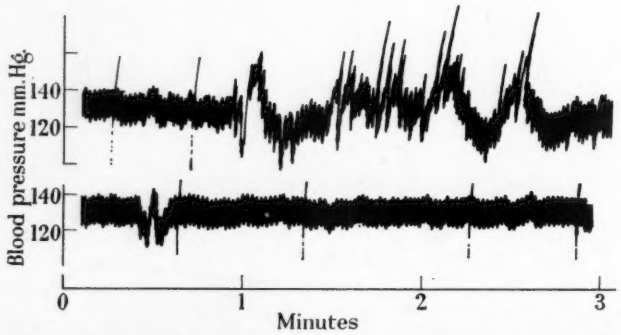

FIG. 5.—Electromanometer tracings of cartoid blood pressure in an unanaesthetised rat. At each signal an indirect reading of blood pressure was made. *Lower trace*, transient disturbance in the record produced by inflating the occluding cuff. *Upper trace :* Violent fluctuations in blood pressure due to struggling. Note the rapid return to normal levels.

During continuous recording of carotid pressure, inflation of the occluding cuff of the tail oscilloscope sometimes caused a small and momentary disturbance of blood pressure in untrained rats (Fig. 5). In more than 90 per cent of cases, however, it had subsided and the blood pressure regained its previous level before the tail reading had been made. On the other hand, if the rat became agitated and struggled, wild fluctuations in blood pressure resulted. Readings obtained on the tail at such times were valueless. It was surprising to find, however, the rapidity with which the blood pressure returned to normal once the animals had ceased to struggle. Usually within 30–60 seconds of the cessation of struggling the blood pressure had returned to its basal level (Fig. 5).

The use of anaesthesia

In describing the use of a tail oscilloscope, Byrom advocated that readings be made under light ether anaesthesia in order to avoid the reported consequences of disturbing the animal. The use of anaesthesia has deservedly fallen into disrepute. Since much important work has been done in the past with the aid of anaesthesia, an attempt has been made to estimate the order of error likely to arise out of its use. Successive indirect readings of blood pressure by tail oscillo-

scope were made during the course of an ether anaesthetic. The mean blood pressures of five such animals are recorded in Fig. 6. These effects of anaesthesia were confirmed while recording directly from the carotid artery. The latter experiments showed in addition that gross fluctuations occurred in blood pressure during the induction and late recovery phases of anaesthesia. It was clear that blood pressure readings were best made between 3 and 5 minutes after commencing the anaesthetic and that readings taken during this period were likely to underestimate the blood pressure by about 10–20 mm. Hg. This underestimate would not be of any great practical importance were it consistent. However, when ether is employed with sick animals or with animals following recent surgery, the underestimates may be large and unpredictable. There is a further disadvantage

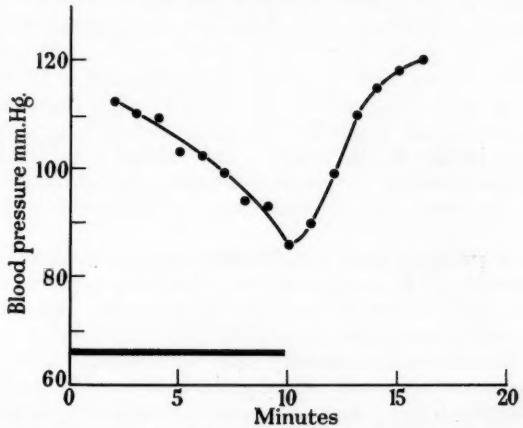

FIG. 6.—Blood pressure recorded by tail oscilloscope during anaesthesia. Each point is the mean of readings on five rats. The duration of ether administration is shown by solid line below.

that serial readings cannot be made under ether anaesthesia. For serial observtions parenteral anaesthetics have often been used. These also suffer from the disadvantage that they may cause an unpredictable depression of blood pressure, especially in the presence of impaired renal function. Moreover, anaesthesia is known to cause modifications of the response of rats to pharmacological agents such as renin (Pickering and Prinzmetal, 1938 ; Scarff and Martin, 1941).

DISCUSSION

The results presented indicate that determinations of blood pressure by the tail oscilloscope correlate very well with simultaneous values of systolic pressure in the carotid artery. For perfect agreement, the slope of the regression line of direct reading on indirect reading should be 1·0. The values calculated from comparisons made on 4 animals gave slopes of 1·03, 0·98, 0·96 and 0·87. There is good reason to believe that the low value (0·87) resulted from a bubble in the tube leading to the manometer. Whether mean pressures or systolic pressures were compared with tail readings, it was clear that the tail reading in any one animal paralleled the carotid reading but at a slightly higher or lower level.

The cause of these small differences in level is obscure. They are as likely to arise from an undetected systematic error in the direct measurement of carotid pressure as from errors in the indirect measurement.

Such accuracy in indirect blood pressure determination can only be achieved if the caudal artery is sufficiently dilated. This is not a peculiarity of blood pressure determinations by the tail oscilloscope or of the caudal artery but a possible source of error in all indirect methods in which a small artery is compressed. Grant and Rothschild (1934), for example, determining blood pressure in the rabbit, found that when the central artery of the ear was constricted blood pressure was underestimated. Moreover these workers demonstrated that in the presence of considerable vasoconstriction there was a 35 mm. drop in pressure along the 5 cm. of the central artery from the base to the tip of the ear.

To ensure that the caudal artery is dilated it is essential that the rat be warmed. Since the heating should be gentle and slow, some objective indication that dilation is adequate is required. A particular advantage of the tail oscilloscopic method of blood pressure determination is that the amplitude of the oscillations picked up from the tail gives satisfactory indication of the presence or absence of vasoconstriction in the caudal artery. The critical amplitude will vary with different tail oscilloscopes but can soon be determined by slowly warming a group of rats and observing the pulse amplitude above which the blood pressure readings form a plateau.

A rise in blood pressure from overheating may be avoided in several ways. A rat may be warmed and as soon as an adequate pulse appears, one or two blood pressure readings taken before it has time to overheat. Alternatively rats can be restrained in wire mesh cages in a temperature-controlled room at 30°–32°. Under these conditions the rats usually have an adequate pulse and their body temperature does not rise unduly. Finally the rats may be warmed in the glass restraint cages described above and a continuous record of their body temperature kept with rectal thermocouples. There is nothing to be said in favour of the first procedure except its speed. It is doubtful that basal conditions can be achieved in this way. The second procedure is more satisfactory but lacks the confidence engendered by monitoring the body temperature.

It is believed that the pressures recorded on rats in the glass restraint cages were basal pressures. Rats were sometimes observed to sleep naturally during this procedure. It was possible to take serial readings in groups of rats for as long as eight hours with only slight fluctuations of blood pressure level. The rats rarely struggled except when, as a result of negligence, they had been allowed to overheat.

To be certain that the blood pressure recorded is basal pressure, it is necessary to take a series of readings over a period. If the readings have a small scatter and do not show trends, it is reasonable to assume their mean is basal pressure. It is true that by taking a series of readings and continuously recording body temperature the determination of blood pressure has become slower and more complicated but this is the penalty required if basal pressures are to be accurately determined.

SUMMARY

A close correlation has been demonstrated between direct measurements of systolic blood pressure and indirect estimates of blood pressure by the tail oscilloscope in the rat. A method has been evolved to enable basal blood pressure

measurements to be made on unanaesthetised rats. With it serial determinations can be made for long periods. Factors which disturb basal blood pressure have been studied.

It is a pleasure to acknowledge our indebtedness to Dr. E. J. Hannan for advice on statistical matters, and to Mr. A. B. Howkins for technical assistance.

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THE PRESSOR RESPONSE TO RENIN DURING PREGNANCY IN THE RAT

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THE blood pressure of rats with experimental renal hypertension falls during pregnancy, often reaching subnormal levels (Harrison, Grollman and Williams, 1940; Wilson and Byrom, 1941; Grollman, 1947). The fall of blood pressure begins about the middle of pregnancy, and is most marked immediately before delivery. The reason for the fall of blood pressure is not known; and indeed the cause of the raised blood pressure of renal hypertension is itself disputed. During the acute phase of renal ischaemia the renin-hypertensin pressor system appears to be implicated (see Goldblatt, 1947); but there is no evidence that it is the only mechanism concerned or that it continues to play a part in the chronic phase of the disease. The sustained hypertension in the latter phase is thought by some to arise from an extra-renal mechanism (Floyer, 1955; Daniel, Prichard and Ward-MacQuaid, 1954). In view of this uncertainty it was of interest to examine the effect of pregnancy on the renin-hypertensin pressor system. In this report the effect of renin on the blood pressure of normal and pregnant rats has been studied.

MATERIALS AND METHODS

Animals.—Female albino rats of Wistar origin were caged with males and segregated when a copulation plug was found. In the virginal state they weighed 150–250 g. Each time a pressor response was measured, a pregnant and non-pregnant animal of similar age and weight were tested simultaneously.

Renin.—Hog renin was extracted as described by Schales (1942). Schales' procedure for concentrating the extract by saturation with NaCl at pH 4·3 was omitted in favour of evaporation from cellophane sacs suspended in a current of air. The final product was lyophile-dried and stored at 5°. Fresh powder was dissolved for each test. The material used was assayed in rabbits (Pickering and Prinzmetal, 1938). One rabbit unit (1·0 R.U.) was the amount of powder required on intravenous injection to cause an average rise of 30 mm. Hg. in the blood pressure of three rabbits weighing 2–4 kg. The renin powder showed no loss of activity during the course of the experiments; it assayed 1·0 R.U./mg. dry weight.

Measurement of pressor response.—Renin injected intraperitoneally produces a blood pressure rise lasting 2–3 hours (Freedman, 1940), and a sustained maximum pressure which is not reached until 20–40 min. after injection. The delayed peak allows the animal to settle down after the injection before the critical readings at the maximum are recorded. Thus the use of anaesthesia, which modifies the response to renin (Braun-Menendez, 1956), was avoided by the intraperitoneal route of injection.

Blood pressure was measured by tail oscilloscope under the basal conditions previously defined (Dodson and Mackaness, 1957). At each test the basal blood pressure was first determined by taking a series of readings at 5-min. intervals until the blood pressure and rectal temperature were steady. The animals were then injected. Subsequent blood pressure readings were made at 3–5 min. intervals until the pressure had returned to normal.

The response to the intravenous injection of renin into a lateral tail vein was also measured in a small number of unanaesthetised animals. Venepuncture was confirmed by withdrawing blood and an interval of 1 or 2 min. was allowed for the animal to settle down before delivering the renin. Blood pressure estimates were then made at ½-min. intervals. The procedure was technically difficult and only occasionally successful.

A comparison of the pressor responses of pregnant and non-pregnant rats would be most revealing if it were based on responses to several dose levels. The development of tachyphylaxis and the changing response of pregnant animals made this impossible. However, it was feasible to demonstrate a progressive change in the response of pregnant animals by making repeated tests at one-dose level. For this purpose a dose producing a maximum

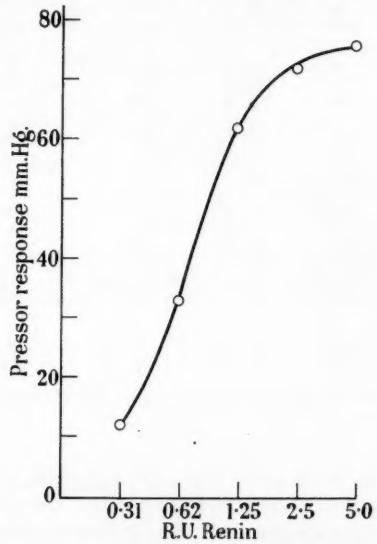

FIG. 1.—Log_2 dose-response curve of the pressor response of normal unanaesthetised rats to intraperitoneal injection of renin. Each point represents the mean response of 3 rats. (R.U. = rabbit unit.)

pressor response was chosen. This was determined from the dose-response relationship of normal rats injected intraperitoneally. Data relating dose and response have been plotted in Fig. 1. It will be seen that a maximum pressor response was achieved with a dose of 5·0 R.U. This was four times the dose required to raise the blood pressure by 60 mm. Hg, and was, therefore, a very large dose of renin.

RESULTS

Comparison between normal and late pregnant rats

The effect of pregnancy on the blood pressure of hypertensive rats is most marked immediately before littering. It was anticipated, therefore, that any effect of pregnancy on the pressor response to renin would be most readily detected at this time. Accordingly, the pressor responses of a group of six pregnant rats were measured on one or more occasions during the last few days of pregnancy. The responses of these animals and of their non-pregnant controls are contained in Table I. It will be seen that in late pregnancy the basal blood

TABLE I.—*Blood Pressure Responses (mm. Hg) to 5·0 R.U. Renin Injected Intraperitoneally in 6 Pregnant Rats before and after Littering and in 6 Non-pregnant Control Rats*

Rat number (litter size)	Days from term	Pregnant: Basal blood pressure	Pregnant: Maximum blood pressure*	Pregnant: Blood pressure rise	Non-pregnant: Basal blood pressure	Non-pregnant: Maximum blood pressure*	Non-pregnant: Blood pressure rise
1	−3	88	119 (7)	31	109	177 (7)	68
(12)	0	73	83 (3)	10	101	179 (6)	78
	+3	123	176 (5)	53	110	184 (6)	74
2	−3	111	141 (6)	30	128	188 (3)	60
(3)	−1	87	99 (3)	12	121	189 (3)	68
	+1	96	170 (3)	74	104	194 (5)	90
3	−4	107	148 (3)	41	122	183 (4)	61
(8)	−2	83	92 (3)	9	110	198 (5)	88
	+2	107	185 (7)	78	119	205 (7)	86
4	−4	101	121 (3)	20	126	194 (4)	68
(10)	−1	65	71 (2)	6	121	190 (5)	69
	+2	109	176 (3)	67	127	200 (4)	73
5	−2	85	99 (5)	14	118	186 (3)	68
(12)	+2	119	191 (7)	72	113	184 (3)	71
6	−2	100	110 (3)	10	121	189 (3)	68
(8)	+2	101	171 (5)	70	119	199 (5)	80

* The maximum blood pressure given is the average of several readings (number in brackets) on either side of the peak pressure.

pressure was lower than in normal animals, and that the pressor response to renin was greatly diminished. After delivery of the litter the basal blood pressure rose towards normal and the response to renin increased markedly. Blood pressure response curves representative of the responses of normal, pregnant and postpartum rats are shown in Fig. 2.

To ensure that this decrease in sensitivity to renin was not a result of delayed absorption or to false intraperitoneal injection two late pregnant rats were injected intravenously. Each received doses of 0·25 and 0·5 R.U. of renin in 0·2 ml. of 1 per cent NaCl. The two dose levels were given with an interval of 4 hours between them. Two non-pregnant rats were injected with the smaller dose. The results, recorded in Fig. 3, confirm in unanaesthetised rats an observation made by Harrison *et al.* (1940) who noted a diminished pressor response when anaesthetised pregnant rats were given an intravenous injection of crude kidney extract. It may be concluded, therefore, that the intraperitoneal route of injection has not been a factor in determining the pattern of responses observed.

The response to renin in relation to the stage of pregnancy

Grollman (1949) has published a curve showing the relationship of the age of pregnancy to the fall of blood pressure in hypertensive rats. A similar curve has been obtained in this laboratory. Both curves show that from about the 10th day of pregnancy the blood pressure of hypertensive rats begins to fall. In rats with large litters it returns to or below normal levels by the 19th or 20th

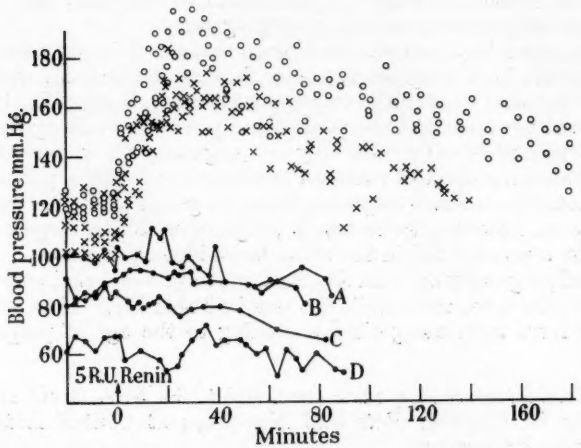

FIG. 2.—Blood pressure readings by tail oscilloscope before and after intraperitoneal injection of 5·0 R.U. of hog renin in 4 non-pregnant rats (○), in 4 pregnant rats before littering (●), and again after littering (×). Curves A and B from rats 2 days ante partum; curves C and D from rats 1 day ante partum.

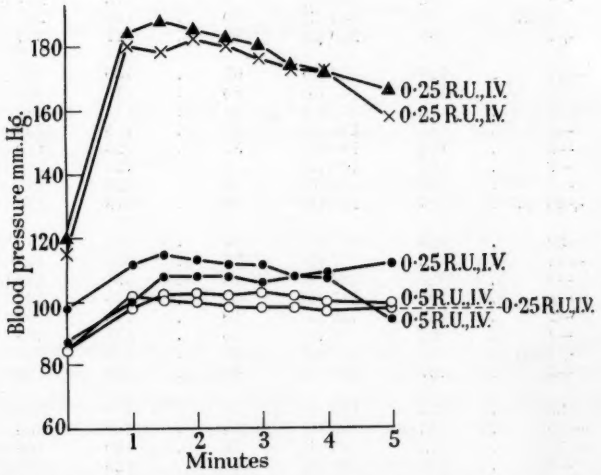

FIG. 3.—Blood pressure readings by tail oscilloscope in two pregnant (●, ○), and two non-pregnant (▲, ×) rats following intravenous injection of renin in the doses indicated. The two pregnant rats received two dose levels of renin at an interval of 4 hours.

day. It was important, therefore, to determine whether the loss of sensitivity to renin was similarly related to the age of pregnancy.

Pressor responses to 5·0 R.U. renin were measured in six rats at intervals throughout the whole of pregnancy. Only four of the animals were pregnant; results from these and their controls are contained in Table II. In Fig. 4 the mean basal blood pressure and the peak blood pressure resulting from the renin injection have been plotted for each day of pregnancy on which a test was performed. Repeated injection of renin at one dose level over a period of 24 days produced a relatively constant response in non-pregnant animals. From the 10th day of pregnancy, however, there was a progressive fall in response which was accompanied by a parallel fall in the mean basal blood pressure.

The effect of pregnancy on both the basal blood pressure and response to renin can be clearly seen from the results set out in Table III. Here all the experimental results have been categorised according to the age of pregnancy at the

TABLE II.—*Blood Pressure Responses (mm. Hg) to 5·0 R.U. Renin Injected Intraperitoneally in Pregnant Rats and Non-pregnant Control Rats at Various Stages during Pregnancy*

Rat number (litter size)	Days from term	Pregnant			Non-pregnant		
		Basal blood pressure	Maximum blood pressure*	Blood pressure rise	Basal blood pressure	Maximum blood pressure*	Blood pressure rise
1	−20	124	195 (4)	71	124	191 (2)	67
(12)	−15	118	199 (5)	81	125	199 (3)	74
	−10	122	187 (4)	65	123	201 (5)	78
	−5	115	150 (6)	35	121	203 (5)	82
	−2	106	117 (3)	11	123	200 (5)	77
	−1	92	101 (6)	9	118	201 (5)	83
	+7 hr.	73	143 (4)	70	119	201 (6)	82
	+2	108	200 (3)	92	121	203 (5)	82
2	−21	128	195 (3)	67	130	189 (2)	59
(8)	−16	117	197 (5)	80	115	208 (6)	83
	−11	112	191 (5)	79	118	195 (3)	77
	−6	121	178 (4)	57	113	204 (4)	91
	−3	112	149 (5)	37	123	200 (8)	77
	−2	102	109 (4)	7	121	200 (5)	79
	−1	88	90 (3)	2	122	197 (3)	75
	+15 hr.	103	169 (4)	66	119	198 (4)	79
3	−20	123	187 (3)	64	132	197 (2)	65
(11)	−14	112	187 (3)	75	123	192 (5)	69
	−8	124	184 (6)	60	121	195 (5)	74
	−4	106	147 (4)	41	114	197 (4)	83
	−2	116	126 (4)	10	125	197 (5)	72
	−1	92	97 (4)	5	118	194 (5)	76
	+3 hr.	91	147 (4)	56	119	197 (5)	78
	+2½	107	156 (7)	49	121	198 (4)	77
4	−20½	124	195 (3)	71	118	186 (3)	68
(12)	−15½	112	196 (6)	84	113	184 (3)	71
	−10½	112	185 (4)	73	110	184 (6)	74
	−5½	112	157 (4)	45	126	194 (4)	68
	−2½	116	126 (5)	10	117	193 (5)	76
	−1½	91	96 (4)	5	117	196 (4)	79
	−½	77	83 (4)	6	112	190 (4)	78
	+1	91	163 (5)	72	116	192 (4)	76

* Number of readings appears in brackets.

time of test. The mean, and standard error of the mean, has been calculated for each group. In the first 10 days pregnant animals did not differ from their non-pregnant controls either in the level of basal blood pressure or in pressor response to renin. During the last two days of pregnancy, however, a maximum pressor dose of renin caused an average blood pressure rise of only 7–9 mm. Hg. Though

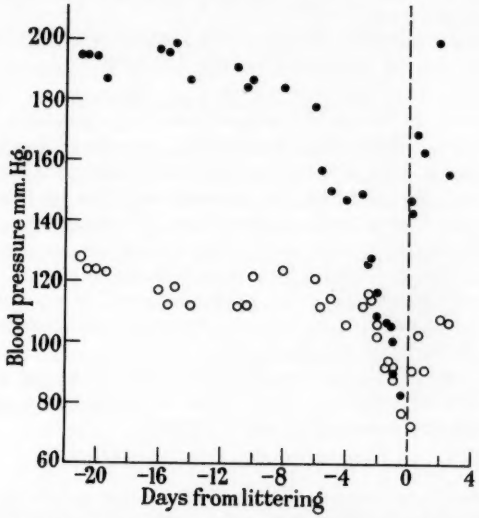

FIG. 4.—Mean basal blood pressure (○), and mean maximum blood pressure (●) following intraperitoneal injection of 5·0 R.U. renin in 4 pregnant rats at various stages during and after pregnancy.

TABLE III.—*Mean, and Standard Error of the Mean, for Blood Pressure Responses (mm. Hg) to 5·0 R.U. Renin Calculated from Data in Tables I and II*

Days from litter date	Number of responses tested	Mean basal blood pressure	Mean maximum blood pressure	Mean blood pressure rise
Controls	48	119±0·9	194±1·1	75±1·1
−11 to −21	9	119±2·0	194±1·4	75±2·3
−1 to −2	7	98±2·0	107±4·6	9±0·4
0 to −1	7	82±3·9	89±4·1	7±0·4
+1 to +2	9	105±2·7	176±4·7	71±3·8

small, this was probably a true response to renin, since in control experiments the injection of saline caused a mean rise of only 2 mm. Hg in the blood pressure of 8 pregnant rats. The prompt reversal of the effect of pregnancy is seen in the average figures obtained for animals tested post partum.

DISCUSSION

These results have revealed two apparently related phenomena : a progressive fall in the basal blood pressure, and a parallel fall in the pressor response to renin during the latter half of pregnancy in rats. It is reasonable to assume that the

basic mechanism involved is the same in each case. The results do not prove that the loss of response to renin and the fall in blood pressure during pregnancy are related as cause and effect. A non-specific loss of vascular reactivity during pregnancy could produce a lowering of basal blood pressure and a loss of responsiveness to pressor agents in general. However, this seems unlikely in view of the evidence in an accompanying paper that pregnant rats respond normally to injected hypertensin (Dodson, 1957).

It has already been mentioned that the mechanism responsible for the rise in blood pressure may not be the same in the acute and chronic phases of renal hypertension. In the rat, whether or not a difference of mechanism exists, the effect of pregnancy is the same : regardless of its duration, pregnancy causes a fall in blood pressure. The effect is identical, except in degree, with that seen in normotensive animals, and hence it corresponds in time with a loss of sensitivity to renin. If the inability of pregnant animals to react with a pressor response is specific to the renin-hypertensin system, the fall of blood pressure in acutely and chronically hypertensive rats may also be due to an inability to react to renin. If this were true it would follow that a renin mechanism operates in both the acute and chronic phases of renal hypertension. Moreover, since the blood pressure also falls in normal rats during pregnancy, renin may have a part to play in maintaining blood pressure at physiological levels.

Experiments to establish the specificity of the changed response to renin during pregnancy are in progress, and in an accompanying paper results of a study on hypertensin are reported (Dodson, 1957).

SUMMARY

The pressor response to intraperitoneal injection of renin has been compared quantitatively in pregnant and non-pregnant rats. Near term pregnant rats are almost totally refractory to the pressor action of renin. Loss of responsiveness begins about the 10th day of pregnancy and is progressive. It is accompanied by a parallel fall in the level of the basal blood pressure. The time-course of development of these changes is identical with the fall in blood pressure of hypertensive rats during pregnancy. A possible relationship between the two phenomena is suggested.

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THE PRESSOR RESPONSE TO HYPERTENSIN IN PREGNANT RATS

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IT has been shown (Mackaness and Dodson, 1957) that during pregnancy rats lose their ability to respond with a rise in blood pressure when injected intraperitoneally or intravenously with maximal pressor doses of renin. The effect of renin on the blood pressure is not direct; it depends upon the enzymic release from a plasma protein substrate of one or possibly more vaso-active polypeptides known as hypertensin. In seeking an explanation for this non-responsiveness to renin, the capacity of the pregnant rat to react to hypertensin is of importance. It distinguishes between a non-specific depression of vascular reactivity and some block to the reaction between renin and hypertensinogen. The present communication describes a comparison of the pressor responses of pregnant and non-pregnant rats to hypertensin.

MATERIALS AND METHODS

Hypertensin.—Hypertensin was prepared with hog renin from bovine plasma by Peart's (1955) method. After elution from charcoal, the dried eluate was taken up in 1 per cent NaCl and neutralised with NaOH. The precipitate which formed was removed by centrifuging and the supernatant was used for test. All experiments were performed on the same batch of hypertensin. Hypertensin is active only by the intravenous route. Its action is so brief that estimates of pressor responses could not be made in unanaesthetised rats with indirect methods of blood pressure estimation. The decision to use anaesthesia was made reluctantly, for it is clear that this introduces factors which modify the pressor responses to hypertensin (*cf.* Braun-Menendez, 1956).

To confirm a preliminary impression that the sensitivity to hypertensin was practically the same in pregnant and non-pregnant rats, the method used for a 6-point bio-assay of hypertensin was adopted and modified. Instead of comparing the responses of a homogeneous group of rats to two different preparations of hypertensin as in a bio-assay, in these experiments a 6-point comparison was made between the responses of the two groups of rats to the same preparation of hypertensin.

Animals.—Albino rats bred in this laboratory from stock of Wistar origin were used. When virgin, they weighed 200–250 g. There were 8 rats in each group. Since the rats were killed after the test, the precise age of the pregnancy could not be determined. Before the experiment it was estimated by abdominal palpation, and subsequently a better estimate was made from the mean foetal weight. The eight non-pregnant female rats were randomly selected from stock of the same age as the pregnant animals.

Anaesthesia.—The tests were performed under urethane anaesthesia. A dose of 0·45 ml. per 100 g. body weight of a 25 per cent (w/v) solution was injected intraperitoneally. It was at first thought possible that the weight gain due to pregnancy might lead to the administration of an effectively larger dose of urethane to the pregnant group. However, reduction of dosage to 0·4 ml. per 100 g. failed to yield an adequate depth of anaesthesia in both pregnant and non-pregnant animals, while an increase in dose to 0·5 ml. per 100 g. appeared to increase the depths of anaesthesia comparably in both groups. So far as could be judged from the effect on blood pressure, respiration, and level of analgesia, the depth of anaesthesia at the dose level used was the same for pregnant and non-pregnant animals.

Recording of blood pressure.—Mean carotid blood pressure was recorded with a small bore mercury manometer (Crawford and Outschoorn, 1951). Heparin (500 I.U.) was used as anticoagulant. The injections of hypertensin were made into a femoral vein with an " Agla " micrometer syringe.

Dose-response curve of hypertensin.—The relationship of blood pressure rise to dose of hypertensin was first determined in a group of normal rats. As reported by Valle, Prado and Prado (1952), the blood pressure rise was a linear function of the logarithm of the dose. With the preparation of hypertensin used in these experiments, log-linear reponses occurred with doses from about 0·0025 ml. to about 0·04 ml. The linear range in any one rat was at least four $\log_2$ doses ; and the range of variation in response between rats to a standard dose of hypertensin was not greater than one $\log_2$ dose level. Blood pressure rises clearly falling within the log-linear part of the response curve lay within the range 15–55 mm. Hg.

Comparison of pregnant and non-pregnant groups.—In order that the range of the 3 doses of hypertensin to be given should be appropriate to the sensitivity of the test animal, a preliminary injection of 0·01 ml. hypertensin was made. If the blood pressure rise thus elicited was relatively large this dose was adopted as the largest of the three given, if small it became the middle dose level. Usually four sets of injections of these three doses of hypertensin were then given in random order.

In most animals the base-line blood pressure and sensitivity to hypertensin remained fairly constant throughout the 12 injections. However, in two of the animals in each group the basal blood pressure and sensitivity to hypertensin declined rapidly following the second or third set of three injections. This loss of sensitivity was attributed to haemorrhage. The subsequent blood pressure responses were not included in the analysis of results.

RESULTS

Examples of pressor responses to hypertensin in pregnant and non-pregnant rats are shown in the Figure. From the pressor responses obtained a mean

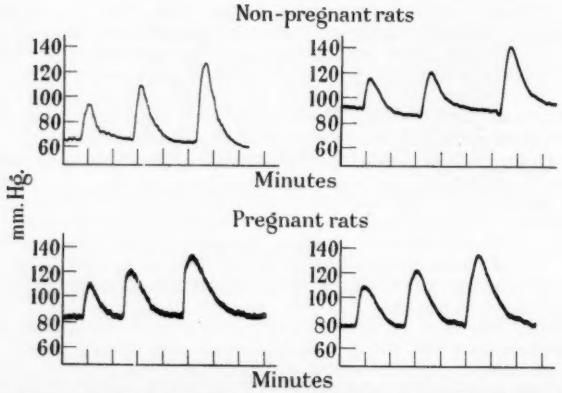

FIGURE.—Pressor responses to hypertensin in pregnant and non-pregnant rats. The tracings on the left were taken from animals receiving intravenous injections of 0·0025 ml., 0·005 ml. and 0·01 ml., and the tracings on the right from animals receiving 0·05 ml., 0·01 ml. and 0·02 ml.

regression line of blood pressure rise on dose was calculated for each rat ; these are set out in the Table.

If it is shown that the parameters a and b of the regression lines for the groups are statistically indistinguishable, it follows that both pregnant and non-pregnant rats give equivalent rises in blood pressure to the same doses of hypertensin.

TABLE I.—*Regression Lines Calculated from the Pressor Responses of Pregnant and Non-pregnant Rats to Doses of the Same Preparation of Hypertensin*

Regression line where
$Y = a + bX$
Y = Rise in B.P. in mm. Hg.
X = Log_2 dose hypertensin

Coding of doses
1 = ·0025 ml.
2 = ·005 ml.
3 = ·01 ml.
4 = ·02 ml.

Rat No.	Dose levels	a	b	Mean weight of foetuses (g.)
	Pregnant group			
1	234	2·67	13·00	2·5
2	234	1·62	10·87	2·5
3	123	9·17	14·25	5·3
4	234	−12·25	12·25	4·8
5	234	−3·08	12·75	5·3
6	234	−11·71	14·12	5·4
7	123	1·68	16·12	6·0
8	123	4·93	15·37	5·9
	Non-pregnant group			
9	234	−4·46	14·62	—
10	123	9·0	12·75	—
11	123	6·83	12·00	—
12	234	−6·25	12·00	—
13	234	−3·46	14·87	—
14	123	9·67	16·50	—
15	123	10·33	13·00	—
16	123	9·51	15·37	—

Estimates of mean regression lines :

Pregnant group	$Y = -0·73 + 13·32\,X$
Non-pregnant group	$Y = 2·67 + 13·47\,X$
Overall	$Y = 0·95 + 13·40\,X$

The " b " coefficients in these three mean regression lines were obtained from the pooled, within-rat, sums of squares and cross products for the relevant groups. Since the " a " coefficients within groups are shown to differ significantly, the " a " coefficients for the three mean regression lines have been obtained as simple averages of the individual " a " coefficients for the relevant sets of rats, these individual coefficients being estimated using the relevant group estimate of the " b " coefficient.

The analysis of variance relevant to the testing of the differences between the slopes of the response lines for each rat is as follows:

Source of variation	D.F.	Sum of sq.	Mean sq.	P
Differences among slopes within groups	14	392	28	·80
Difference between pregnant and non-pregnant groups	1	2	2	·96
Variation about individual regression lines	160	6544	41	—
Total	175	6938	40	

Clearly there are no significant differences either between individual slopes within either of the two groups or between the average slopes for the groups.

For the levels of the responses " a " we have the following analysis :

Sources of variation	D.F.	Sum of sq.	Mean sq.	P
Differences among levels of response within groups	14	8735	624	·001
Difference between levels for two groups	1	46	46	—

The F ratio for testing within groups difference is

$$F14, 160 = \frac{624}{41} = 15{\cdot}2$$

which of course is highly significant so that there is no doubt that the level of response differs from rat to rat, within the same group. The relevant comparison for the between groups effect is now

$$F1, 14 = 0{\cdot}1 \qquad P - 0{\cdot}8$$

so that there is no evidence of any significant change in the level of response between the two groups. The average level of response is higher for the non-pregnant group but the difference is small relative to the variations in level between rats in the same group. Almost all the within-groups difference in levels is due to differences between the levels for the two sequences of doses but this probably means merely that the technique for choosing the dose sequence efficiently separated out the low response from the high response rats. The equivalence of the responses of the two groups may be illustrated in another way. The mean regression lines computed for each group could be used to predict the average blood pressure rises resulting from the injection of hypertensin. If the dose of hypertensin, predicted to yield a 50 mm. Hg rise in blood pressure in the normal group were injected into the pregnant group, it would cause an average rise of 46 mm. Hg.

DISCUSSION

The lowest mean foetal weight in the Table is 2·5 g. which corresponds to the 19th–20th day of pregnancy. Hence all the pregnant rats tested were within the period of maximum depression of response to renin.

It could be argued that the blood pressure fails to rise following injection of renin because of an increased capacity of pregnant animals to destroy hypertensin ; and that the rises of blood pressure occurring after its injection are due to rapid introduction of hypertensin in amounts sufficient to swamp even an augmented inactivating mechanism. If this were the explanation for the difference in response to renin and hypertensin, it would still be expected that an appreciable blunting of the response to hypertensin could be detected in pregnant rats. The shape of the pressor response curve was, however, identical in pregnant and non-pregnant animals (Figure). Moreover, the peak response to intravenous injection of renin occurs within 1½ minutes, so that the reaction time *in vivo* for the release of hypertensin by renin is not much longer than the time required for mixing. It seems likely, therefore, that the ability to respond to hypertensin, but not to renin, cannot be due to more rapid destruction or inactivation of hypertensin in pregnant rats.

Another possibility is that a species difference exists between hypertensin elaborated by the rat and the hypertensin prepared in the manner described above. There is some evidence that species differences exist in the amino acid composition of hypertensin (see Peart, 1956). There is no evidence, however, that hypertensin prepared from different sources show pharmacological differences.

These experiments were carried out with anaesthetised rats and it is generally agreed that anaesthesia depresses the response to pressor agents. For anaesthetic effects to invalidate the comparison of pressor responses between the two groups

would require that the non-pregnant rats suffered a greater anaesthetic depression than the pregnant. There is no evidence for such a differential effect of anaesthesia. Since the pregnant rats received larger absolute amounts of urethane, the vaso-depressive effects of anaesthesia might be expected to be greater on these.

Assuming that the foregoing objections are not valid the present experiments have the following implications. Late in pregnancy rats fail to give significant rises in blood pressure when injected with very large doses of renin. That this is not a non-specific depression of vascular reactivity is shown by their normal response to injected hypertensin. Moreover, renin is not directly pressor, and its pressor activity is mediated by liberation of hypertensin. It follows that in pregnancy the injection of renin fails to liberate hypertensin in an active pressor form. This could result from a variety of causes. There may be inadequate amounts of renin substrate in the blood of pregnant rats. Renin may be rapidly destroyed or it may fail to react because of the presence of an inhibitor. Renin may be inactivated by the developing foetal kidneys, although the work of Page (1947) does not support this notion. There may be a failure of the conversion of hypertensin I to hypertensin II if the converting enzyme described by Skeggs, Marsh, Kahn and Shumway (1954) is absent. Some of these possibilities are susceptible to investigation.

SUMMARY

A quantitative comparison has been made of the capacity of pregnant and non-pregnant rats to respond with blood pressure rises to the intravenous injection of hypertensin. In late pregnancy the pressor response to hypertensin is normal. This contrasts with the greatly reduced pressor response previously found in late pregnant rats when renin is injected. Possible explanations of these findings are discussed.

It is a pleasure to acknowledge my indebtedness to Dr. E. J. Hannan for unstinted help in the statistical treatment of data, to Dr. T. S. B. Robertson and Dr. G. B. Mackaness for the preparation of hypertensin used and to Mr. A. B. Howkins for technical assistance.

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A TWO-STAGE METHOD OF CROSSLINKING PROTEINS SUITABLE FOR USE IN SEROLOGICAL TECHNIQUES

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IN an earlier publication (Coombs, Howard and Mynors, 1953) a technique was described which was theoretically capable of detecting incomplete antibodies to soluble protein antigens. The method involved the crosslinking of a soluble protein antigen to rabbit anti-bovine cell serum using tetrazotised benzidine and sensitizing "inagglutinable" bovine red cells with this conjugate to form a stable red-cell-protein-antigen complex. This was then used to adsorb antibodies to the soluble protein antigen whence the complex could be agglutinated by building an anti-globulin lattice in a similar manner to that described by Coombs, Gleeson-White and Hall (1951).

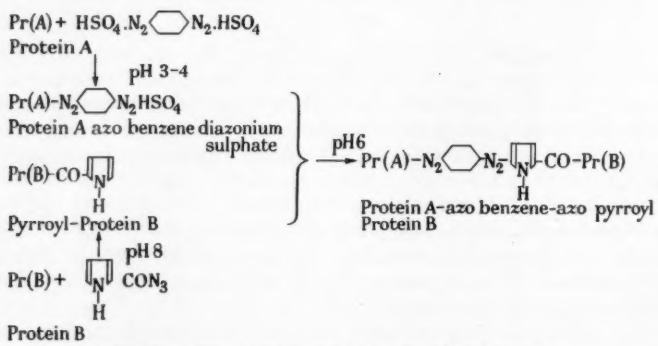

FIGURE.—Two-stage method of cross-linking proteins.

It is clear that in the initial crosslinking reaction using tetrazotised benzidine, coupling occurs between protein molecules other than those of the specific protein and antibody. In this present publication a two-stage method of crosslinking proteins is described in which the combination between the two different proteins is specific. The technique depends on the unusual properties of tetrazotised *p*-phenylenediamine and pyrrole in acid solution. Namely, that one of the functional groups of tetrazotised *p*-phenylenediamine will couple with phenolic groups in acid solution whereas the second group will couple only in alkaline media (Shoutissen, 1933); also that pyrrole derivatives in contrast to phenols are capable of coupling with a single diazonium group in acid solution (Fischer and Hepp, 1886). The basis of the crosslinking method is illustrated in the Fig.

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Protein A was allowed to react with tetrazotised *p*-phenylenediamine at pH 3 to pH 4, reaction being expected by virtue of the phenolic groups in the protein, and the compound formed was protein A containing free diazonium groups (protein A benzene diazonium hydrogen sulphate). Phenolic and other groups are thought not to react at this pH value. The protein complex after purification by dialysis, was allowed to react at pH 6, with protein B which had been conjugated with pyrrole. Crosslinking between protein A and protein B was expected by virtue of the reaction of the free diazonium groups of the protein A complex with the pyrrole groups of conjugated protein B.

For the success of this method it was necessary to conjugate pyrrole to protein B. This was achieved by the reaction of protein B with pyrrole-α–carboxylic acid azide in weakly alkaline media, a similar technique to that used in the conjugation of aspiryl groups to horse and rabbit globulins by Butler, Harington and Yuill (1940).

To illustrate the two-stage method of crosslinking proteins, egg albumin which had been conjugated with tetrazotised *p*-phenylenediamine was reacted with rabbit anti-bovine-cell serum conjugated with pyrrole in the manner outlined above. The preparation after dialysis was tested for the presence of crosslinked proteins by the lattice technique described in the earlier paper (Coombs *et al.*, 1953). In a second method, bovine red cells were sensitized with rabbit anti-bovine cell serum conjugated with pyrrole, and the cells after washing were reacted in the cold with egg albumin conjugated with tetrazotised *p*-phenylenediamine. Crosslinking of the rabbit anti-bovine cell antibody on the cells with egg albumin was then detected by the lattice technique referred to above.

MATERIALS AND METHODS

Egg albumin was prepared by Hopkins' method described by Cole (1933). Rabbit anti-bovine red cell serum and rabbit anti-globulin sera were obtained as described by Coombs *et al.* (1953). Bovine red cells of the inagglutinable class were used in the testing (Coombs, *et. al.*, 1951). Pyrrole-α-carboxylic acid azide was prepared by the method of Piccinini and Salmonini (1902) from pyrrole-α-carboxylic acid methyl ester (Ciamician and Silber, 1884).

The conjugation of rabbit anti-bovine cell serum with pyrrole acid azide

To 2·5 ml. rabbit anti-bovine cell serum was added 10 mg. pyrrole acid azide in 0·3 ml. ether and 100 mg. sodium bicarbonate. The reaction mixture was shaken at room temperature from 1½ hr. and dialysed against 0·1 M phosphate buffer, pH 7, for three days. The preparation was kept at −20° until required.

Tetrazotised p-*phenylenediamine*

Recrystallized *p*-phenylenediamine (0·54 g.) was dissolved in 17·5 ml. syrupy phosphoric acid with warming. This solution was cooled to −5° in an ice salt mixture, and a cold solution of 0·72 g. sodium nitrite in 7·5 ml. conc. sulphuric acid added very slowly over 20 min., keeping the temperature at −5°. After standing at −5° for half an hour, 0·25 g. urea was added in order to remove the excess nitrous acid. The preparation was used immediately the effervescence had subsided.

Conjugation of egg albumin with tetrazotised p-*phenylenediamine*

A conjugation on a basis of 5 moles tetrazotised *p*-phenylenediamine per mole of protein (assuming the molecular weight of egg albumin to be 40,000) was effected as follows. The above tetrazo-reagent (0·125 ml.) was added to water (1 ml.) at 5° followed by 1 ml. saturated sodium acetate solution. This mixture was added immediately to 4 ml. ice cold 5 per cent egg albumin in saline and allowed to stand half an hour at 5°, the acidity of the reaction

mixture being pH 3·5. The preparation was dialysed overnight at 5° against 0·1 M sodium acetate buffer pH 4·0, and either used immediately or stored at −20° for a few days.

RESULTS

Crosslinking of Egg Albumin with Rabbit Anti-bovine Cell Serum

To 1·25 ml. rabbit anti-bovine cell serum conjugated with pyrrole acid azide was added, dropwise at 5°, 0·75 ml. egg albumin conjugated with tetrazotised *p*-phenylenediamine, the final reaction being at pH 6. The mixture was allowed to stand at 5° for some hours and dialysed for 48 hr. against saline.

A 2 per cent suspension of "inagglutinable" bovine red cells in 0·1 ml. amounts was added to equal volumes of a series of progressive dilutions in saline of rabbit anti-bovine cell serum conjugated to egg albumin. After 30 min. incubation at 37°, the cells in each tube were washed and exposed to a 1/20 dilution of guinea-pig anti-egg albumin serum. After 30 min. at 37° each tube was centrifuged, the cells washed in saline and finally resuspended in 0·1 ml. saline. The detector system then used was identical to that of "Stage C" as described under "Methods" in our earlier publication, Coombs *et al.* (1953).

The results are shown in Table I. Agglutination was detected up to a titre of 80, indicating that the expected crosslinking of egg albumin to rabbit anti-bovine cell serum had occurred. The titre was however lower than that obtained by the one-stage method using tetrazotised benzidine (Coombs *et al.*, 1953), the results of which are also quoted in the table.

TABLE.—*Conjugation of Egg Albumin to Rabbit Anti-bovine Cell Serum: Comparison of Two-Stage Method with the One-Stage Method*

Preparations and controls*	Dilutions of sensitizing system									
	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 256	1 : 512	1 : 1024	Saline
1 .	++	++	++	++	++	++	++	++	−	−
2 .	++	++	++	++	−	−	−	−	−	−
3 .	++	++	++	−	−	−	−	−	−	−
4 .	−	−	−	−	−	−	−	−	−	−
5 .	−	−	−	−	−	−	−	−	−	−

Rabbit anti-guinea-pig globulin (1 : 40) was final antisera used in detecting sensitization as described in Coombs *et al.* (1953).

Key: ++ = strong agglutination.
+ = definite but weak agglutination.
− = no agglutination.

* 1.—One-stage method using tetrazotised benzidine.
2.—Two-stage method: serum and egg albumin conjugated prior to addition of cells.
3.—Two-stage method: cells sensitized with rabbit anti-bovine cell serum conjugated with pyrrole azide with subsequent addition of egg albumin conjugated with tetrazotised phenylene diamine.
4.—Two-stage method control: cells sensitized with untreated rabbit anti-bovine cell serum and subsequent addition of egg albumin conjugated with tetrazotised phenylenediamine.
5.—Control: normal rabbit serum.

Crosslinking of Egg Albumin to Rabbit Anti-bovine Cell Antibody

To 0·25 ml. of a 2 per cent suspension of washed "inagglutinable" bovine red cells was added 0·25 ml. rabbit anti-bovine cell serum, conjugated with pyrrole acid azide, diluted progressively with saline. The tubes were incubated for 30

min. at 37°, centrifuged, and the deposited cells washed twice in saline. After being resuspended in 0·15 ml. 0·2 M phosphate buffer, pH 7·0; the cells were cooled in ice. To each tube was added 0·075 ml. egg albumin conjugated with tetrazotised *p*-phenylene diamine and the suspensions left at 5° for 30 min. with occasional shaking. The cells were centrifuged, washed with saline, and exposed to a 1/20 dilution of guinea-pig anti-egg albumin serum. The detector system was again that described under "Methods" in the paper by Coombs *et al.* (1953). The titre obtained was 20 (Table) which compares with 80 for the preceding experiment. In a control experiment, untreated rabbit anti-bovine cell serum was used instead of that conjugated with pyrrole acid azide. No agglutination was observed (Table) in this case indicating that conjugation of pyrrole to the rabbit serum was essential for crosslinking to take place.

DISCUSSION

The use of tetrazotised benzidine for the crosslinking of proteins has been described previously (Coombs, Howard and Wild, 1952; Coombs *et al.*, 1953) but the method is of limited value as it is non specific. The crosslinking of two different proteins has been carried out by a two-stage coupling reaction and the success of the coupling has been shown by the agglutination technique of Coombs *et al.* (1953).

SUMMARY

A two-stage method for crosslinking proteins suitable for use in serological techniques is described. One protein is reacted with tetrazotised *p*-phenylenediamine in acid solution; the other with pyrrole-α-carboxylic acid azide in weakly alkaline solution. On reaction of these two preparations at pH 6 crosslinking of the two proteins occurs. The method is theoretically more specific than that using tetrazotised benzidine.

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THE ACTION OF DIPHTHERIA TOXIN ON TISSUE CULTURES AND ITS NEUTRALIZATION BY ANTITOXIN

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IT has been known for a considerable time that tissue cultures are susceptible to the action of diphtheria toxin. Levaditi and Mutermilch (1913*a*, 1913*b*) showed that the fibroblastic growth from chick embryo fragment cultures, made by the hanging drop technique, was inhibited by diphtheria toxin and that this action was neutralized by specific antitoxin. These findings were subsequently confirmed by others, including Okabe (1933) whose results suggested that such cultures might be of value in titrating dipheria antitoxin. Using similar methods Lasfargues and Delaunay (1946) studied the action of diphtheria toxin on cultures of guinea-pig embryo but these proved only moderately susceptible.

With the new methods of tissue culture which have been developed during the last few years in the field of virology, it has now been possible to make a more precise study of the cytotoxic action of diphtheria toxin and its neutralization by antitoxin.

MATERIALS AND METHODS

Cytotoxic test

The majority of tests were made with monkey kidney cell cultures prepared by the method described in the recent report from the Biological Standards Control Laboratory (1957). Trypsinized cells were suspended to a concentration of approximately 200 thousand per ml. in Parker 199 medium containing 5 per cent horse serum free from diphtheria antitoxin. The suspension was distributed in 1 ml. quantities into 6 × $\frac{5}{8}$ in. Pyrex test tubes which were held at 37° in an almost horizontal position for 6 to 7 days when confluent cell sheets were formed. Before using the cultures they were twice washed, each time with 1 ml. of Hanks' solution, and then fed with 1 ml. of Parker 199 medium containing 0·14 per cent bovine albumin (Armour's fraction V). After inoculation with 0·2 ml. of toxin or 0·4 ml. of toxin-antitoxin mixture, the cultures were incubated at 37° in roller machines and examined 4 days later for cytotoxic changes under the low-power microscope.

pH colour test

In the few tests made by the pH colour method, which was adapted from the one described by Salk, Younger and Ward (1954) for titrating poliomyelitis antibodies, cell suspensions were prepared from the confluent sheets of monkey kidney cell cultures by stripping them off the glass with a 0·2 per cent solution of versene. The cells were suspended to a concentration of 2 × 10^5 per ml. in Hanks' solution containing 0·5 per cent lactalbumin hydrolysate, 2 per cent diphtheria antitoxin-free horse serum and phenol red as indicator. The suspension was distributed in 0·5 ml. quantities into 3 × $\frac{1}{2}$ in. Pyrex test tubes and then inoculated with 0·2 ml. of toxin or 0·4 ml. of toxin-antitoxin mixture. After inoculation, 0·5 ml. of liquid paraffin was layered on to the surface of the liquid in each tube. The tubes were then incubated stationary in a vertical position at 37° and examined 4 days later for inhibition of cell growth which was indicated by the colour of the phenol red.

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Toxins

Three diphtheria toxins were used, numbers 2776, 3092 and 3095 with Lf values of 30 200 and 140 respectively. The toxins were diluted in Hanks' solution for all tests.

Antitoxins

Tests were made with the British Standard Diphtheria Antitoxin (10 units per ml.) and three antitoxins, numbers 1071, 576, and 653, prepared in horses. None of the sera contained preservative. The antitoxins were diluted in Hanks' solution for all tests.

Determination of the minimal cytotoxic dose

Two-fold dilutions of toxin were made and from each dilution six tissue culture tubes were inoculated, each with 0·2 ml. The cultures were examined after incubation at 37° for 4 days and the minimal cytotoxic dose (m.c.d.) taken as the highest dilution of toxin causing disruption of the cell sheet and destruction of more than 50 per cent of cells.

Determination of the test dose of toxin

Dilutions of toxin with 10–20 per cent differences were made and 2 ml. of each dilution added to 2 ml. of standard antitoxin containing 0·2 unit. The mixtures were held at room temperature for 1 hr. and from each mixture at least four tissue culture tubes were inoculated, each with 0·4 ml. The titration was read after 4 days at 37° and gave the smallest dose of toxin which, with 0·02 unit of antitoxin, produced cell destruction. This test dose is referred to as the Lc/50.

Titration of antitoxin

Dilutions of antitoxin were made at 25 per cent differences and 2 ml. of each dilution added to an equal volume of toxin containing 10 times the Lc/50 test dose. With the same dilution of toxin, a titration of the standard antitoxin was always made as control. The mixtures were held at room temperature for one hr. and from each at least four culture tubes were inoculated, each with 0·4 ml. The neutralizing dose of antitoxin was determined after 4 days, incubation and the potency of the serum expressed as units of antitoxin per ml.

In vivo *titrations*

Titrations of toxin and antitoxin were also made in the skin of guinea-pigs by the classical method of Römer.

EXPERIMENTAL AND RESULTS

The Action of Toxin on Cell Cultures

The action of toxin on monkey kidney tissue cultures was first evident 24 to 48 hr. after inoculation. The cell sheets became disrupted and the majority of cells necrotic and detached from the glass. Some islets of cells, however, remained attached to the glass and were not grossly altered morphologically, although they appeared enlarged with pronounced granularity of the cytoplasm. The picture of degeneration differed from that produced by the cytopathic action of, for instance, poliomyelitis virus, in which islets of unaltered cells are seldom present and the necrotic cells have a more shrunken appearance.

Occasionally with toxin 2776, the undiluted toxin did not act as rapidly as some of the higher dilutions although degeneration became complete in three to four days. This phenomenon was not investigated but it was probably due to some inhibitory constituent of the broth in which the toxin was contained.

In most tests Parker 199 medium was used to maintain cell growth but the toxin acted equally as well on cultures which were maintained in Hanks' solution containing 0·5 per cent lactalbumin hydrolysate.

Each of the three toxins was titrated in cell cultures and in the skin of guinea-pigs. It is evident from the results (Table I) that the two tests showed a similar order of sensitivity. There was no difficulty in reading the end-points of the titrations in cell cultures, as may be seen from the results of a typical test in Table II.

TABLE I.—*The Activity of Three Toxins in Monkey Kidney Cell Cultures and in the Skin of Guinea-pigs*

Toxin no.	M.C.D. tissue culture (ml. $\times 10^{-7}$)	M.R.D. guinea-pig (ml. $\times 10^{-7}$)
2776	25	25
3092	2·5	2·5
3095	1·25	2·5

TABLE II.—*The Titration of Toxin* 2776 *in Monkey Kidney Cell Cultures*

Toxin in inoculum (ml. $\times 10^{-7}$)	Proportion of cultures showing complete degeneration
100	6/6
50	6/6
25	5/6
12·5	0/6

Monkey kidney cell cultures were also shown to be susceptible to the action of toxin by the pH test; the m.c.d. of toxin 2776 was similar to that obtained by the cytotoxic test.

The cytotoxic activity of toxin was destroyed by heat; dilutions of toxin containing 500 m.c.d. per ml. when heated at 58° for 2 hr. showed no action on cell cultures.

Neutralization of Toxin by Antitoxin

Test dose of toxin

Table III gives the results of the determination of the test dose of each of the three toxins against 0·02 unit of antitoxin in monkey kidney cell cultures (Lc/50) and in the skin of guinea-pigs (Lr/50). The values obtained by the two methods were very similar, indicating again that the two tests were of the same order of sensitivity. Furthermore, titrations in cell cultures gave sharp end-points, as may be seen from the results of a typical titration given in Table IV.

TABLE III.—*Test-doses of Three Toxins Determined in Monkey Kidney Cell Cultures and in the Skin of Guinea-pigs*

Toxin no.	Test dose (ml.) in cell cultures Lc/50	Test dose (ml.) in guinea-pigs Lr/50
2776	0·0007	0·001
3092	0·0001	0·0002
3095	0·0001	0·0002

Titration of antitoxin

The three antitoxins, 1071, 576 and 653, were titrated in cell cultures in parallel with the standard antitoxin, using the Lc/50 test dose of toxin 2776. The test dose contained approximately 280 m.c.d. and, on account of this, clear cut end-

TABLE IV.—*The Determination of the Lc/50 Test-dose of Toxin* 2776

Dose of toxin (ml.) mixed with 0·02 unit antitoxin	Proportion of cultures showing complete degeneration
0·0009	9/9
0·0008	9/9
0·0007	9/9
0·0006	0/9
0·0005	0/9

points were obtained as may be seen from the results of a typical titration given in Table V. The results obtained for each antitoxin by both the cell culture and the guinea-pig method are given in Table VI and it is evident that there was good correlation between the two tests, although with each antitoxin the values obtained in cell cultures were approximately 30–50 per cent higher than those in guinea-pigs.

TABLE V.—*The Titration of Antitoxin* 576 *in Monkey Kidney Cell Cultures Using Toxin* 2776

Mixtures of		Proportion of cultures showing complete degeneration
Antitoxin (ml.)	Toxin (ml.)	
1/10,000	0·0007 = Lc/50	0/4
1/12,500		0/5
1/14,200		0/5
1/16,600		4/5
1/20,000		5/5
1/25,000		5/5

TABLE VI.—*Antitoxic Potency of Three Antitoxins Determined in Monkey Kidney Cell Cultures and in the Skin of Guinea-pigs*

Antitoxin no.	Units/ml.	
	Cell culture method	Guinea-pig method
1071	240–280	170
576	280–330	230
653	1540–1900	1350

Titration of antitoxin by the pH colour test

The Lc/50 test dose of toxin 2776 was also determined by the pH colour test. The end-point was taken as the smallest dose of toxin which, with 0·02 unit of antitoxin, produced complete destruction of cells as indicated by the red colour of the culture fluid. The Lc/50 value of the toxin was found to be similar to that obtained by the cytotoxic test. A titration of the standard antitoxin was made by this method (Table VII) and, although no other antitoxins have yet been tested, the result indicates that the method should be of value.

Characteristics of the Cytotoxic Action.

Two tests were made in parallel in which 500 m.c.d. of toxin 2776 diluted in 1 ml. of Parker 199 medium was left in contact with cell cultures for different periods of time. The culture tubes were incubated at 37° in roller machines and

TABLE VII.—*The Titration of Standard Antitoxin in Monkey Kidney Cell Cultures by the pH Colour Method*

Mixtures of Antitoxin (units)	Toxin (ml.)	Colour in each culture
0·016	0·0007 = Lc/50	R R R R R R
0·018		R R R R R R
0·020		R R R R R R
0·022		P P P P P P
0·024		O O O O O O
0·026		Y Y Y Y Y Y

R = Red. P = Pink. O = Orange. Y = Yellow.

for each period a group of 11 tubes was used. In one test, the fluid was removed at the end of each period and each culture washed twice, each time with 5 ml. of Hanks' solution, the fluid being replaced with 1 ml. of Parker 199 medium. In the second test, the fluid was removed at the end of each period, the cultures washed twice as before and 1 ml. of Parker 199 medium containing 300 units of antitoxin added to each culture. After treatment the cultures were incubated at 37° in roller machines and examined at four days. The results are shown in the figure.

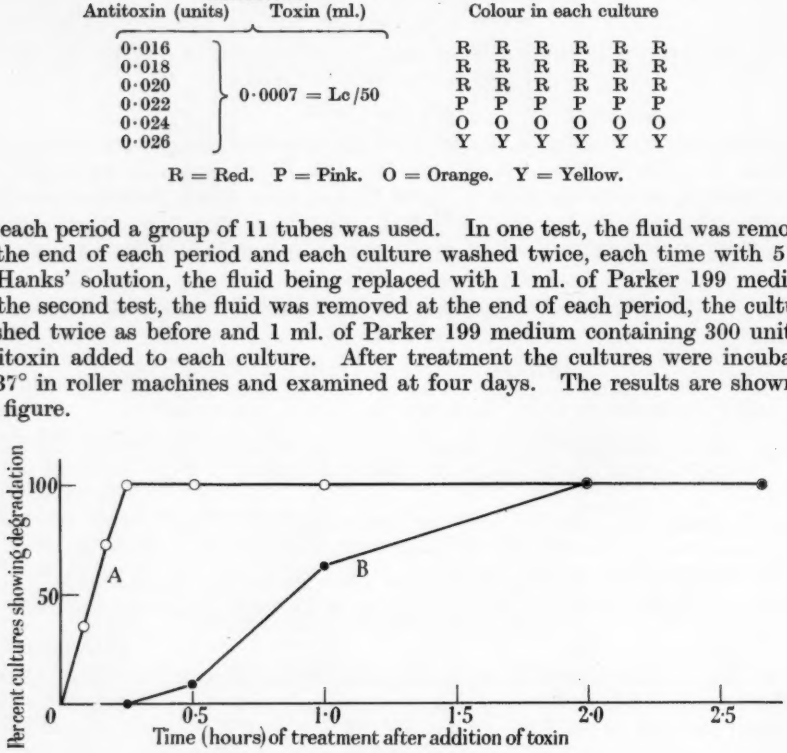

FIGURE.—The effect on cell cultures of :

A. washing
B. washing + antitoxin

at varying intervals after the inoculation of toxin.

It is evident that 500 m.c.d. of toxin was able to initiate cytotoxic action in all tubes after contact with cells for 15 min. since washing the cells after this time was unable to alter the course of cell destruction. Antitoxin, however, proved to be more effective than washing, for when added to cells within 2 hr. after the addition of toxin it was able to inhibit cytotoxic action but when added later it was completely ineffective.

DISCUSSION

The results of this investigation have shown that monkey kidney cell cultures are as sensitive to the action of diphtheria toxin as the skin of guinea-pigs and suggest that such cultures may be of value in studying the mode of action of

toxin under controlled conditions. Other cell cultures may be equally satisfactory and we have already found that HeLa and human amnion cell cultures are of the same order of susceptibility to diphtheria toxin as monkey kidney cultures.

It has been shown that when 500 m.c.d. of diphtheria toxin is added to monkey kidney cell cultures the toxin is soon absorbed by the cells but that its toxic action does not occur immediately on absorption since the later addition of antitoxin is able to protect the cells from destruction. If, however, antitoxin is withheld for longer periods it is then unable to reverse the intoxication process. These findings are similar to those obtained by Miles (1949) who studied the fixation of diphtheria toxin in the skin of guinea-pigs. He concluded that when toxin is injected intradermally there is at first a fixation that is reversible by antitoxin but that later an irreversible fixation occurs, the precursor of intoxication, which antitoxin is unable to modify.

Cell cultures may also be of value in titrating the potency of antitoxic sera. With each of the three antitoxins tested, the culture method gave slightly higher potency values than the guinea-pig method. This difference may be explained on the basis of the dissociation of the toxin-antitoxin complex which is less likely to occur in cell cultures than in the skin of guinea-pigs, and the culture method may in fact give a truer value of antitoxic potency. If this should prove to be the case, then it is possible that the culture method may be highly suitable for the titration of low potency sera which, as Long (personal communication) has shown, give irregular results when titrated in guinea-pigs.

SUMMARY

Monkey kidney cell cultures are as susceptible as the skin of the guinea-pig to the action of diphtheria toxin. HeLa and human amnion cell cultures are also susceptible to the toxin.

The cytopathic action of diphtheria toxin in monkey kidney cell cultures is neutralized by specific antitoxin. The antitoxic potency of sera may be titrated using cell cultures as indicator.

Diphtheria toxin acts quickly on cell cultures. There is at first an absorption which is reversed by the addition of antitoxin but not by washing the cultures. This is followed by a fixation of toxin which leads to cell destruction and is irreversible by antitoxin.

We are indebted to Miss Mollie Barr for supplies of toxin and antitoxin.

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SURVEY OF PAPERS

FILDES and KAY describe a bacteriophage that is active on a wide range of *Salmonella typhi* strains. Under natural conditions it required trytophan. Experimentally they found it possible to select a series of mutants that were almost entirely indifferent to trytophan (p. 563).

ELEK and CONEN used human volunteers to determine the virulence of *Staph. pyogenes*. No differences were detected using strains from lesions, healthy nasal carriers or epidemics. Intradermal, subcutaneous, scarification and incisional inoculations showed no differences in numbers of cocci necessary to give rise to infection. Adjuvants failed to increase the virulence. The presence of a foreign body reaction greatly increased susceptibility to infection (p. 573).

WESTWOOD and TITMUSS describe stable transformed and untransformed cell lines derived from the same monkey kidney tissue culture stem and also a cell line in which spontaneous reversion from the transformed to the untransformed state occurred. The chromosome numbers of all the cell lines examined lay in the hypo-tetraploid region but the authors regard the acquisition of polyploidy as a secondary process which is not essential to adaptation. They failed to demonstrate *in vitro* the inhibition of polyploid by diploid cells which is presumably required by the view that transformation depends on the release of small numbers of polyploid cells from suppression by the diploid stock. Transformation could be simulated by environmental modifications but the changes induced reverted as soon as normal conditions were restored. A hypothesis is advanced to explain the known phenomena of transformation (p. 587).

WARDLAW reports that the bactericidal activity of human, rat and guinea-pig sera against *Bact coli* and *Sh. shigae* is greater when diluted with *clinical* dextran than when diluted with saline. This potentiating effect, which contrasts with the inhibitory effect of *native* dextran, is related to molecular weight, constitution and concentration of the dextran. The mechanism is unknown but appears not to depend on agglutination or increased sensitivity of the organisms, or protection or potentiation of complement (p. 601).

BOYDEN presents evidence of interference in the guinea-pig between the responses to tuberculo-protein and tuberculous infection. In contrast to the controls, 11 days after BCG vaccination guinea-pigs which had received tuberculo-protein four to five weeks before vaccination failed to react to intradermal tuberculin (p. 611).

DODSON and MACKANESS describe a tail oscilloscope method for measuring the basal blood pressure of unanaesthetized rats, and discuss the factors which influence the values obtained. The figures given by this method agreed closely with direct measurements of carotid artery blood pressure (p. 618).

MACKANESS and DODSON show that the basal blood pressure of pregnant rats falls rapidly during the last few days of pregnancy and rises rapidly post-partum. These changes, which parallel those seen in hypertensive animals, are accompanied by a fall followed by a rise in the responsiveness to renin. A possible relationship between these findings is suggested (p. 628).

DODSON has shown in the rat that late in pregnancy, when the response to renin is greatly reduced, the response to hypertensin is normal. The contribution of this finding to the understanding of the response to renin is discussed (p. 635).

HOWARD and WILD describe a method of crosslinking two different proteins which is suitable for use in serological techniques. Only one diazo group of tetraazotised p-phenylenediamine will react with the phenolic groups of the first protein in acid solution leaving the remaining diazo group to form the cross-link to the second protein which reacts as a protein-pyrrole complex. Egg albumin has been crosslinked to rabbit anti-bovine cell serum and to rabbit anti-bovine cell antibody (p. 640).

PLACIDO SOUSA and EVANS have studied the effect of diphtheria toxin upon monkey kidney tissue cultures. They show that the tissue cultures are as susceptible as guinea-pig skin, that the cytopathic action of the toxin can be neutralised by antitoxin and that the antitoxic potency of sera may be titrated using tissue cultures as indicated (p. 644).

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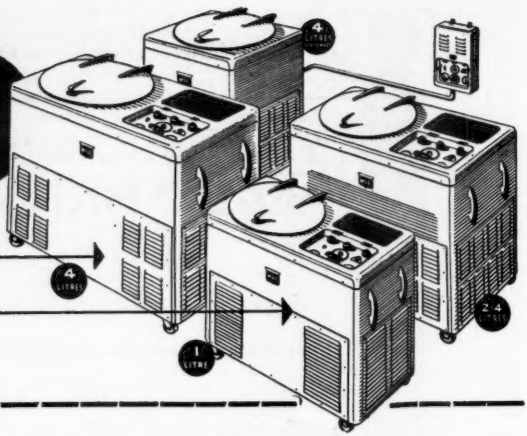

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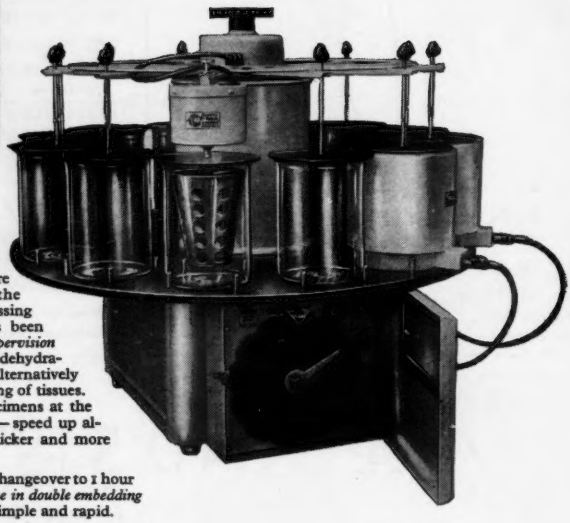

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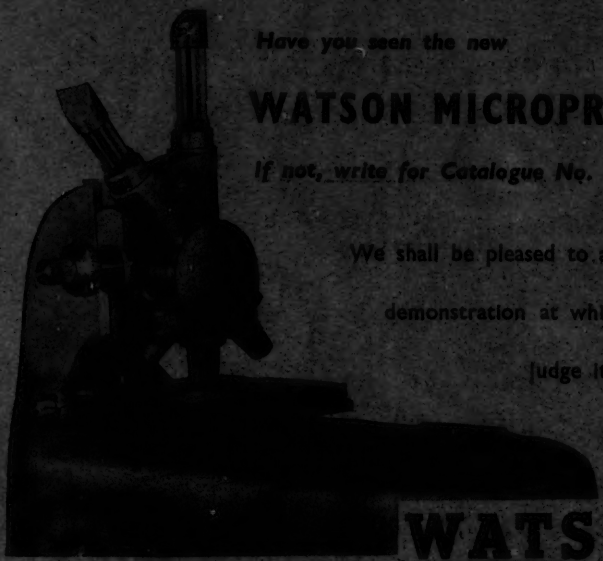

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